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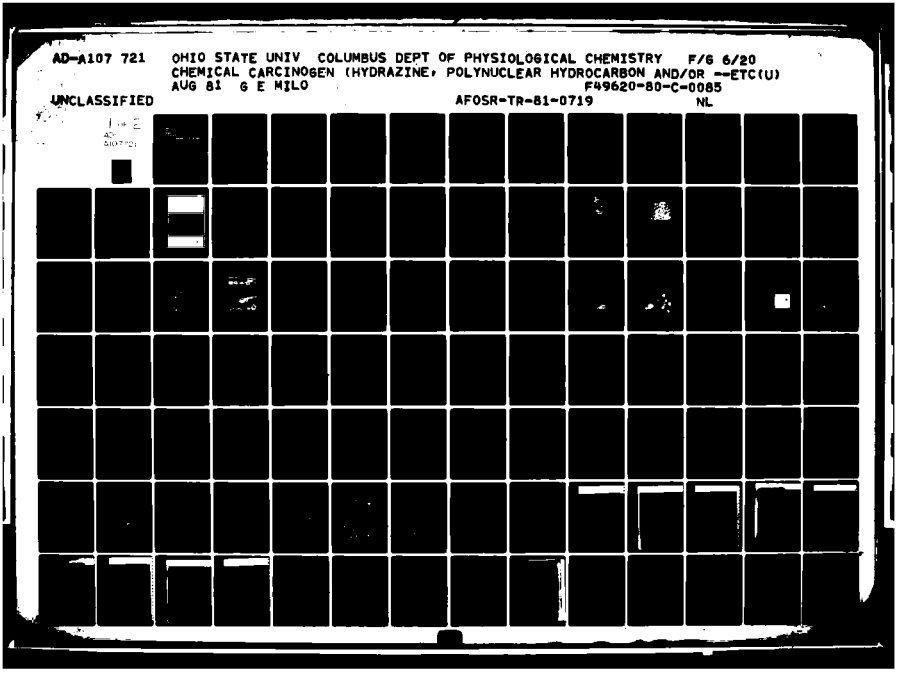

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Progress Report

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CHEMICAL CARCINOGEN (HYDRAZINE, POLYNUCLEAR HYDROCARBON AND/OR SYNTHETIC JET FUEL COMPONENTS) INDUCED CARCINOGENESIS OF HUMAN CELLS, IN VITRO

George Milo
Department of Physiological Chemistry

For the Period
August 1, 1980 - August 31, 1981

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
Bolling Air Force Base, D.C. 20332
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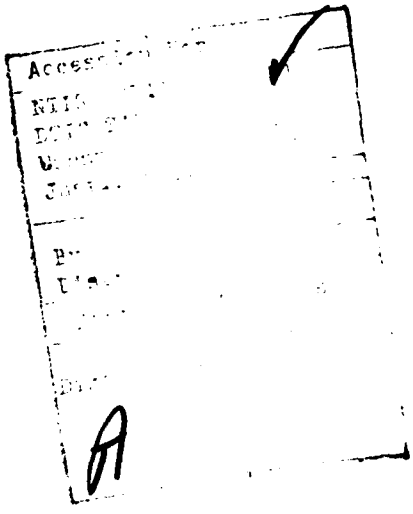
20. Abstract

Select chemicals of interest to the Air Force and identified as man made environmental contaminants have been found to neoplastically transform human cells in vitro. Benzo(a)pyrene one of these compounds of interest, exhibits unique features of movement from the extracellular environment to the nucleus of the cell. B[a]P is transported into human foreskin fibroblasts from the plasma membrane to the nucleus by binding as the unmetabolized parent B(a)P. B[a]P forms a low binding association with a lipoprotein complex in the cytoplasm that has a MW of 12,500. The B[a]P following penetration into the nucleus is then oxygenated to various metabolites. Benzo pyrene-diol-epoxide-I has been identified as a metabolite in the nucleus that interacts with deoxy-Guanine to form a DNA adduct, 7R-BPDE-I-dG.

We then used another hydrocarbon 1,2,3,4-tetrahydro-7,12-dimethyl benz(a) anthracene to induced carcinogenesis in human cells. To date we have no evidence for extracellular or intracellular metabolism of this compound under conditions for transformation. We conclude tentatively that this data suggests that there is an alternate requirement for polynuclear hydrocarbon induced carcinogenesis in human cells compared to rodent cells.



Progress Report to

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

Directorate of Life Sciences

Bolling A.F.B., D.C. 203332

Title: Chemical Carcinogen (Hydrazine, Polynuclear Hydrocarbon and/or Synthetic Jet Fuel Components Induced Carcinogenesis of Human Cells, In Vitro

Inclusive Dates of Report:

August 1, 1980 to August 31, 1981

Submitted by: George E. Milo

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The experiments reported herein were conducted according to the principles described in "Guide for the Care and Use of Laboratory Animals" prepared for the Committee on Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 78-23, revised 1928.

George E. Milo

George E. Milo Ph.D.
Comprehensive Cancer Center

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i) **Work Scope 1980-1981**

a) To investigate how UDMH, HZ and/or BP are transported to the cell nucleus

b) To investigate changes in DNA directed DNA polymerase I and II activity following treatment with UDMH, HZ and/or BP

c) To examine changes in histone labeling patterns during the early and late stages of the carcinogenic process following exposure to UDMH or BP

d) To investigate the interaction of HZ and BP metabolites with DNA during the induction process and correlate this with the metabolic profiles in the activation stage

e) To develop a predictable and reliable procedure using human cells in vitro to evaluate the carcinogenic potential of chemicals of interest to the air force

ii) **Progress 1980-1981**

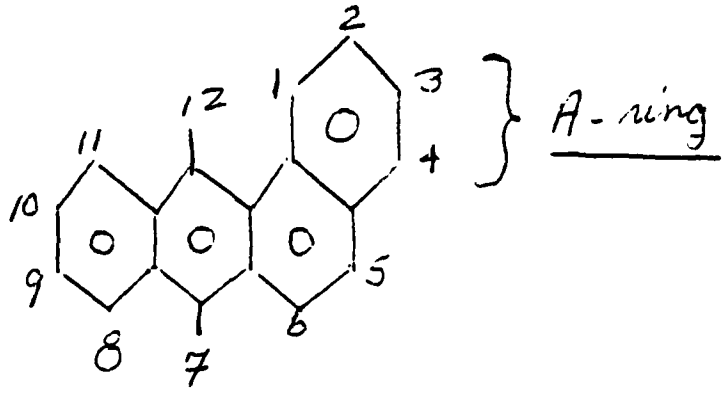
We found that BP was transported to the nucleus as the parent BP via a cytoplasmic protein complex. This cytoplasmic complex had a molecular weight of 12,500 MW, Tejwani et al. 1980. Analysis of the intracellular distribution and binding of benzo(a)pyrene in human diploid fibroblasts, was expected in Cancer Lettters 10:57-65. Since these experiments were completed we have isolated the complex and separated the 12,500 MW complex into subfractions on a potassium bromide gradient, (p, 1.006-1.279) at 200,000 x g for 24 hrs. In excess of 30% of the recovered radiolabel was associated with low density lipoprotein (p, 1.073) and 25% of the radiolabel was associated with high density lipoprotein fraction (p 1.125), (Fed Amer. Soc. of Exp Biology 40, 1577, 1981).

The penetration of the BP into the nucleus required energy and the transport across the nuclear membrane is temperature dependent. When the temperature of the cultures is dropped from 37°C to 4°C BP is not found in the nucleus. Isolation of the nuclei from the BP treated cells incubated at 4°C revealed that no radiolabel was present in the nuclei. Under conditions for transformation we found that BP localized in the nucleus was oxygenated and 7,8,9/10 ene diol Benzo(a)Pyrene anti form of the Benzo Pyrene ene diol epoxide (BPDE-I) was produced. It appears as though the BP is oxygenated in the nucleus possibly in the chromatin area. (Submitted to Carcinogenesis 1981).

Treatment of the cells with BPDE-I under conditions for transformation resulted in a transformed phenotype. DNA-adduct analysis of the BPDE-I interaction with the DNA of transformable and non-transformable cells (Collaborative studies with Dr. Alan Jeffery Comp. Cancer Center Columbia College of Physicians and Surgeons, N.Y., N.Y.) illustrated that the 7R-BPDEI-dG in the transformable cells was the only adduct that change in quantity, i.e. the amount of 7R-BPDEI-dG decreased 80% in the non-transformable populations.

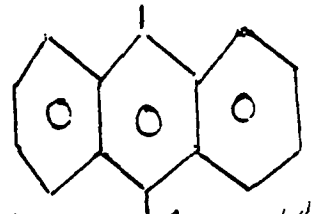
When we examined the transformable and non-transformable populations treated with BP the nuclear base-adduct that changed in the non-transformable cells was the 7R-BPDEI-dG adduct. In a recent series of experiments we added BP to confluent dense cultures and observe that greater than 40% of the BP in the extracellular medium was metabolized to oxygenated metabolites. However, there was no radiolabel found in the nuclei of these cultures. These cultures could not be transformed following BP treatment nor were there any oxygenated metabolites found in the nuclei of these treated cultures, (Tejwani and Milo, Cancer Res. 1981 In Press).

To examine the involvement of particular rings of the polynuclear hydrocarbons; the lipophilicity of the active carcinogen was examined; we decided to look at 7,12-dimethylbenzanthracene as a prospective candidate because of the arrangement of the aromatic nuclei.



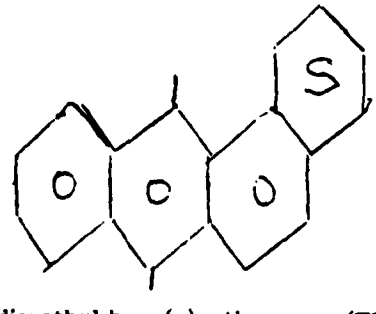
7,12 - DMBA

Reduction of the A ring left the dimethyl anthracene nucleus as the unconjugated system.



dimethyl anthracene

This compound when applied to human cells was non-carcinogenic to very weakly carcinogenic. With a reduced hexene ring then the compound appears as the following structure:



1,2,3,4-tetrahydro-7,12-dimethyl benz(a)anthracene, (THDMBA).

The THDMBA treated cells had a transformation index of 168; 7,12 DMBA treated cells had a transformation index of 0; BP treated cells had a transformation index of 1; 9,10-dimethyl cyclo penteno a anthracene treated cells had a transformation index of 23. The transformation index was calculated as the number of colonies containing 50 cells or more per colony per 10^5 cells seeded into 0.33% agar in a 25 cm^2 well, (Tejwani et al., Cancer Letters 13, 119-127, 1981). The saturation of the A ring in the DMBA prevented the DMBA from being oxygenated in the bay region or K-region. These are the reported regions for epoxides to be formed that lead to the penultimate forms of oxygenated polynuclear hydrocarbon carcinogenic metabolites.

We have recently had synthesized through NCI the radiolabeled derivatives of the tetrahydro form. We plan to examine the profile of possible metabolites by high performance liquid chromatography.

iii) **Principal Experiments in Progress with Other National Laboratories**

We are presently concluding work with Dr. Fred Kadlubar at the National Center for Toxicological research on the activity of the amines and their deterivatives on the interaction of the amines and DNA. Another collaboration that will bear fruition presently will be our work with Dr. Allen Jefferies at the College of Physicians and Surgeons Comprehensive Cancer Center at Columbia University. We are examining the relationship between chemical carcinogen induction and adduct formation. Our work with Dr. Donald Witiak is beginning to bear results. We are assisting his laboratory in two ways. First, we are studying the relationship between the hydrazine compounds and derivatives with the induction of carcinogenesis and adduct formation. Secondly, we are using the radiolabelled hydrazine carcinogens in our system to study the events that occur during the expression phase of carcinogenesis. This is being accomplish by studying the interaction of the hydrazines with the acid soluble histone and non-histone nuclear proteins. We have concluded the radiolabeling studies and are finishing up the studies designed to quantitate the changes in histones during the early and transitional stages of the carcinogenesis processs.

The work with Dr. Kadlubar on aromatic amines has been accepted for publication in Carcinogenesis, (1981). We have completed the evaluation of the neoplastic potential of the transformed cells on the chick embryonic skin system as a suitable substitute for the mouse. This work has been accepted for publication in In Vitro, (1981). We have completed the transformation of human skin epithelial cells and the manuscript is in Press, (Cancer Res 1981).

We are continuing to work on the interactions of HZ and UDMH and BP with the cell nucleus. Both in collaboration with Dr. Witiak and Dr. Trewyn. (Objective f-1981). Dr. Tomei is working on the Promoters and sensitizers and the effect they have on the preinduction process. (Objective g-1981). His report is as follows.

1) Human foreskin fibroblasts were determined to be resistant to mitogenic stimulation by animal tumor promoting phorbol esters following cell cycle arrest by amino acid deprivation, serum deprivation, and diminished Ca^{++} concentrations. These results are in contrast to results obtained and reported by Tomei et al (1980, 1981a, 1981b) using the murine embryonic fibroblast C3H-10T1/2. These findings are inconsistent with the concept of skin tumor promotion based upon animal data.

2) Human foreskin epithelial cells were studied with respect to responses to putative tumor promoting phorbol esters.

a. Cell cycle activation following arrest in both serum deficient and nutrient deficient media was not observed. Increased nuclear labeling could, however, be induced when ^{3}H-thymidine specific activities exceeded 35 Ci/mmole. It was observed that this labeling (autoradiography) approached 50-90% of the cells after 24 hrs treatment and was markedly uniform with respect to grain number. Furthermore, it was observed that regions of heavy cornification had diminished grain counts. It suggested two critical modifications of future experimental protocols; ^{3}H-thymidine specific activities be kept below 10 Ci/mmole and blockade of radioactive missions be prevented by keratin removal prior to preparation of autoradiography. In subsequent experiments using low specific activity ^{3}H-thymidine and epithelial cells stripped of overlaying keratin, TPA was not observed to be mitogenic. This was confirmed using another non-phorbol mouse tumor promoter, benzoyl peroxide.

b. Mouse tumor promoting phorbol ester, TPA, was observed to have a profound effect on patterns of cornification in normal epithelial islets. Short term experiments (24-72 hr exposure) revealed that TPA enhanced keratinization determined by both Mallory histological staining and epifluorescence obtained with rhodanile blue (see below). This was in marked contrast to long term experiments (5-15 days) which revealed that large, uniform islets developed having markedly diminished total keratin as determined by histological staining.

Conclusions based upon these observations are consistent with the concept that tumor promoters act on regulatory processses modifying the patterns of cell differentiation. It is believed that basal cells are inhibited from progressing through keratinization, whereas, cells that have progressed beyond an initial commitment stage are accelerated through to maturation and release into a cornified upper stratum. This is believed to be related to modulation of carcinogenic transformation as described previously by Milo and co-workers and the possible relationships are being investigated.

c. Epifluorescent determination of keratinization of substantially improved reliability was developed. Using the stain rhodanile blue (RB) which stains glycoproteins, proteins and cytoskeletal components, it was found that water elution produced marked variations in staining densities and characteristics between culture dishes. This has been discussed by others as a serious shortcoming of their stain. However, it was found that methanol elution of cells stained with 1% RB in water resulted in the visible loss of approximately 90% of the staining density. Since it was known that RB absorbed strongly in the green light region and had an extremely strong red fluorescence, the methanol eluted cells were examined using epifluorescence techniques by exciting the cells with green light and observing fluorescent emission in the red region.

It was found that cytoskeletal components were strongly fluorescent, whereas, cytoplasmic and nuclear fluorescence was markedly diminished. Since a major component of the cytoskeleton in human skin epithelial cells is keratin, results were compared with results obtained with fluorescent antibody techniques using rabbit anti keratin and fluorescene coupled goat anti-rabbit sera. Thus far, these techniques have revealed that basal layers of epithelial cells have substantial variations in keratin levels and cytoplasmic organization. Furthermore, these variations are not uniform but rather form clusters implying local cellular regulation (see Tomei, J. Theoretical Biol. in press).

II. Investigation in progress:

(1) Cell cycle effects of tumor promoters in skin fibroblasts are being examined following G_1 "staging" (i.e. pretreatment of cells with peptide hormones, steroid hormones, and antioxidants during serum deprivation stage of G_1 blockade).

(2) It is suspected that lung fibroblasts (human embryonic) will have distinctly different responses to phorbol esters, presumably involving cell cycle activation without a requirement for pretreatment.

(3) Another marker of tumor promoter activity has been related to induction of trypsin resistant adhesion. Using ^{3}H-fucose (a 5 carbon carbohydrate), prelabelled cells, it has thus far been found that TPA inhibits incorporation of the radiolabel ^{3}H from the medium into the cytoskeleton by 15-25% over 48 hrs in the absence of glucose in the extracellular medium.

We are currently examining the ability of TPA to increase the fraction of detergent-resistant ^{3}H-fucose containing glycoproteins. It is known that TPA substantially increased the resistance of skin fibroblasts to triton X-100 disruption.

Further experiments are in progress to determine responses of lung-derived fibroblasts obtained from primary human mammary and mammary tumor cultures.

(4) Collaborative efforts are underway with Dr. Ernest Kuhn (U.C.S.F.) to determine poly ADP ribose polymerase activities in skin and lung fibroblasts and the effects of pretreatment with mouse tumor promoting phorbol esters, non-phorbol esters and anti-oxidant agents.

Objective H

We have several papers in preparation on metabolite formation by skin fibroblast and skin epithelial cells, see enclosed manuscripts. This work is continuing. We have completed radiolabeling synthesis of the reduced A ring 7,12-DMBA and we are presently investigating its possible metabolite profile using the radiolabelled compound.

iv. Papers Published in 1980 Sponsored by A.F.O.S.R.

1. G. Milo, R. Olsen, S. Weisbrode, and J. McCloskey (1980) Feline sarcoma virus induced in vitro progression from premalignant to neoplastic transformation of human diploid cells. In Vitro. 16:813-822.

2. G. Milo, S. Weisbrode, R. Zimmerman and J. McCloskey. (1981) Ultraviolet radiation induced neoplastic transformation of normal human cells in vitro. Chem. Biol. Int. 45-59.

3. G. Milo and J. DiPaolo (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis. Internat. J. of Cancer. 26, 805-812.

4. Milo, G., G. A. Ackerman, and I. Noyes, 1980. Growth and ultrastructural characterization of proliferating keratinocytes in vitro without added extrinsic factors. In Vitro 12:20-30.

5. Tejwani, R., S. Nesnow and G. Milo 1980. Analysis of intracellular distribution and finding of benzo(a)pyrene in human diploid fibroblasts. Cancer Letters, 10:57-65.

6. Noyes, I., G. Milo and C. Cunningham, 1980. Establishment of proliferating human epithelial cells in vitro from cell suspensions of neonatal foreskin. Tissue Culture Assoc. Lab. Manual 5:1173-1176.

7. Milo, G., and J. Di Paolo, 1980. Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis. Int. J. of Cancer. 26:805-812.

8. Milo, G., R. Trewyn, R. Tejwani, and J. Oldham, 1980. Intertissue variation in benzo(a)pyrene metabolism by human skin, lung and liver, in vitro. Advisory group for aerospace research and development. NATO. Toxic Hazards in Aviation. B7-1 to B7-9.

V. Papers to be Published in 1981 Sponsored by AFOSR

1. Milo, G., S. Weisbrode, R. Zimmerman, and J. A. McCloskey, 1981. Ultraviolet radiation induced neoplastic transformation of normal human cells. Chem. Biol. Int. 36:45-49.

2. Milo, G., I. Noyes, and J. Donohoe, and S. Weisbrode, 1981. Neoplastic transformation of human epithelial cells in vitro after chemical carcinogen treatment. Cancer Res. In Press.

3. Milo, G., J. Oldham, R. Zimmerman, G. Hatch, and S. Weisbrode, 1981. Characterization of human cells transformed by chemical and physical carcinogens, in vitro. In Vitro, In Press.

4. Tejwani, R., Witiak, D., Inbasekaran, N., Cazer, F., and G. Milo, 1981. Characteristics of benzo(a)pyrene and A-ring reduced 7,12-DMBA induced neoplastic transformation of human cells in vitro. Cancer Letters 13:119-127.

5. Tejwani, R., Trewyn, R., and G. Milo, 1981. Kinetics of movement of Benzo(a)pyrene into transformable and non-transformable human diploid fibroblasts. Symposium on polynuclear aromatic hydrocarbons. Batttelle Memorial Inst. Vth ed. 97-107.

6. Tejwani, R., Jeffrey, A., and G. Milo, 1981. Benzo(a)pyrene diol epoxide DNA adduct formation in transformable and non-transformable human foreskin fibroblast cells in vitro. Carcinogenesis. Submitted.

7. Tejwani, R., and G. Milo. 1981 Metabolism of benzo(a)pyrene in transformable and non-transformable human skin fibroblast cells. Cancer Res. Submitted.

8. Donahoe, J., Noyes, I., Milo, G., and S. Weisbrode, 1981. A comparison of expression of anchorage independent growth with neoplasia carcinogen transformed human fibroblast in nude mouse and chick embryonic skin in vitro. In Vitro. In Pres..

9. Cazer, F., Inbasekaran, N., Loper, J., Tejwani, R., Witiak, D., and G. Milo, 1981. Human cell neoplastic transformation with benzo(a)pyrene and A-Bay region reduced analogued of 7,12-dimethylbenz(a)-anthracene. Symposium on polynuclear hydrocarbons. Battelle Memorial Inst. Vth ed. 499-506.

vi. **Presentations, Abstracts - FY - 1980**

June 1980 Tissue Culture Assoc., St. Louis, Missouri. Establishment of human lung epithelial cells in vitro.

June 1980 Amer. Soc. Biol. Chemistry BP metabolism by transformable human skin fibroblasts.

Sept. 1980 NATO International Toxicology Symposium Toronto, Ontario.

Sept. 1980 Battelle Memorial Inst. Polynuclear Hydrocarbon Symposium. Kinetics of Movement of BP into the nucleus of transformable cells.

ATTACHMENTS

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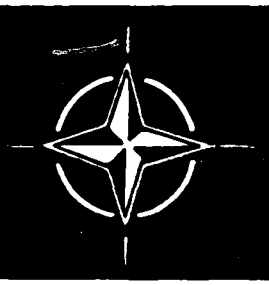

DISTRIBUTION AND AVAILABILITY

INTERTISSUE VARIATION IN BENZO(a)PYRENE METABOLISM BY HUMAN SKIN, LUNG AND LIVER IN VITRO

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Running Title: Human Cell B(a)P Metabolism

SUMMARY

Benzo(a)pyrene [B(a)P], an environmental carcinogen, has been shown to transform human skin fibroblasts in vitro. This fossil fuel combustion product and other polynuclear hydrocarbons have exhibited a requirement to be biotransformed to their ultimate carcinogenic forms to induce transformation. B(a)P diol-epoxides are the most cited candidates as ultimate carcinogens.

When proliferating skin epithelial cells (Phase III type cells) were treated with ^{3}H-B(a)P, 83% was extractable into the organic phase as B(a)P while in the non-proliferating cells 61% occured as the parent B(a)P. Human Lung epithelial cells and liver hepatocytes were also treated with ^{3}H-B(a)P and the metabolites analyzed by HPLC. Patterns of metabolism of B(a)P by these cell populations were dissimilar to those for skin epithelial cells. In lung and liver only 9.2-21.1% remained as unmetabolized B(a)P in the non-proliferating cells, whereas 26.4% remained as B(a)P in the proliferating lung cells.

Proliferating low passage human skin fibroblasts were treated for 24 hours with ^{3}H-B(a)P after which the metabolites were removed from the growth medium with ethyl acetate. Analysis of the organic phase by HPLC demonstrated that B(a)P-tetrols (diol epoxides), B(a)P-diols and B(a)P-phenols represented a small portion (2% ea.) of the metabolites.

Our results suggest that either the ultimate form of the carcinogen is different for fibroblasts and epithelial cells or the quantitative generation of hydroxylated metabolites is not required for neoplastic transformation in fibroblast cells. If hydroxylation is required, then the site of hydroxylation may be the significant factor. Present evidence suggests that in B(a)P treated fibroblasts the activation of B(a)P in the cell takes place other than the microsomal P450 complex, presumably in the nucleus. In epithelial cells from primary target tissues, the microsomal P450 complex may play a more predominant role in the carcinogenesis process compared to the fibroblast microsomal complex.

ABBREVIATIONS

B(a)P-Benzo(a)Pyrene; MEM-minimum essential medium-Eagle; FBS-fetal bovine serum; [G-^{3}H]-B(a)P-tritium-labeled benzo(a)pyrene; Ci-curie; BHT-butylated hydroxytoluene; HPLC-high performance liquid chromatography; PDL-population doubling; NFS-neonatal foreskin; HEL-human embryonic lung

INTRODUCTION

The metabolism of the environmental carcinogen benzo(a) pyrene [B(a)P] occurs in animal tissues through several pathways intended for detoxification, i.e. the mixed function oxidases or by conjugation with polar groups either sulfates, glucuronic acid or glutathione. These pathways yield a mixture of organic and water soluble metabolites, many of which have been implicated as ultimate carcinogenic forms of B(a)P (1-6).

Among the primary target tissues of B(a)P-induced carcinogenesis are the skin, lung and liver, either due to direct exposure (skin, lung) or due to an ultimate role in detoxification (liver). Metabolite profiles of B(a)P produced by cells in culture originating from any of these organs would be very informative. Since most cancers are of epithelial origin (carcinomas), the metabolism of B(a)P by epithelial cells placed in culture from these target organs would be of particular interest.

B(a)P metabolism studies are available for several rodent species (7-10). Due to the concern over human exposure, however, B(a)P metabolite profiles from human cells would be more desirable (11,12,13,14). In this report, we describe the in vitro biotransformation of B(a)P by human epidermis, peripheral lung epithelial cells and liver parenchymal cells in vitro. We compared also the B(a)P metabolite profiles of human epithelial cells with human [illegible]

MATERIALS AND METHODS

MATERIALS

[G-^{3}H] B(a)P (19 Ci/mmole) was purchased from Amersham Searle, Arlington Heights, IL. Synthetic B(a)P metabolite standards were received through the Chemical and Physical Carcinogenesis Branch, National Cancer Institute, Bethesda, MD.

Eagle's Minimum Essential Medium (MEM) containing 25 mM Hepes buffer was purchased from Grand Island Biological Co., Grand Island, NY. MEM-25 mM Hepes at pH 7.2 was supplemented with sodium pyruvate (1.0 mM), glutamine (2.0 mM), nonessential amino acids (1X) and vitamins (1X) (15). All of these supplements were obtained from M.A. Bioproducts, Walkersville, MD). The MEM also contained sodium bicarbonate (0.2%) and gentamycin (5ug/ml).

Fetal bovine serum (FBS) was purchased from Reheis Chemical Co., Kankakee, IL. Collagenase was obtained from Worthington Biochemical Corp., Vineland, NJ, Instagel scintillation cocktail from Packard Instrument Co., Downers Grove, IL, and methanol (MCB OmniSolv) for HPLC from Curtin Matheson Scientific, Inc., Cleveland, OH.

METHODS

Primary Skin Epithelial Cell Cultures

Primary cultures of human neonatal foreskin epithelial cells were established as described previously (16-18). This method involves an initial digestion of the tissue with collagenase (16), followed by selective detachment of fibroblasts with trypsin after the primary culture was established (17,18).

Primary Lung Epithelial Cell Cultures

Human fetal lung tissue obtained from William J. Douglas (Tufts University) was enzymatically dispersed for 4 hours with 0.25% collagenase in MEM supplemented with 20% FBS. The digestions were done at 37 C in a 4% CO_2 enriched air environment. The cells were centrifuged at 650 x g for 10 minutes, washed with MEM, and resuspended in MEM containing 20% FBS. The cells were then seeded at a concentration of 20,000 cells/cm^2. After 2 hours, the residual lung cells in suspension were removed, and the cells attached to the substratum were fed with MEM containing 40% FBS.

The primary lung cell cultures contained less than 10% fibroblasts when the B(a)P metabolism studies were undertaken. They were composed of mixtures of epithelial cell types. However, the major portion of the population contained lamellar structures, tonofilaments, and desmosomes as determined by electron micrsocopy. At this time, we have designated these cultures as mixed peripheral lung epithelial cell populations.

Primary Liver Parenchymal Cell Cultures

Normal adult human liver was obtained from the Tumor Procurement and Pathology Laboratory, Comprehensive Cancer Center, Ohio State University. Liver from surgery was placed immediately into culture by a modification of the method of Schaeffer and Kessler (19). The tissue was minced into 1 to 2 mm pieces and incubated at 37 C for 6 hours in the presence of 0.25% collagenase in MEM containing 20% FBS. Following the incubation, FBS was added to a final concentration of 50% and the parenchymal cells were selectively pelleted by centrifugation at 650 x g for 3 minutes. The cells, were resuspended in MEM containing 20% FBS and insulin (0.5 U/ml), and were seeded into 25 cm^2 tissue culture flasks at a concentration of 15,000 cells/cm^2. Following a 4 hour attachment period at 37 C in a 4% CO_2 enriched air environment, the cultures were washed to remove debris. The cultures were fed with MEM containing 20% FBS, and a confluent primary culture of parenchymal cells was obtained within 72 hours. The cultures were comprised of greater than 95% liver parenchymal cells.

Primary Skin Fibroblast Cell Cultures

Human neonatal foreskin fibroblasts were grown, serially subpassaged and treated with B(a)P as described previously (2).

[G-^{3}H] B(a)P Treatment

Treatment of cells with [G-^{3}H] B(a)P (0.105uM at 1 mCi/ml, 19 Ci/mmole) was accomplished after dissolving the compound in spectral grade acetone. The final solutions were added to MEM containing 10% FBS at 37°C. The [G-^{3}H] B(a)P-containing medium (3.3 ml/25 cm^2 flask) was used to feed the cultures, after which they were incubated at 37°C in a 4% CO_2 enriched air environment (20). All procedures with [G-^{3}H] B(a)P were carried out under yellow light.

B(a)P Metabolite Extraction

Twenty-four hours after the administration of radiolabeled B(a)P, aliquots of the growth medium were removed and partitioned with 3 volumes of ethyl acetate containing the antioxidant butylated hydroxy toluene (BHT, 0.8 mg/ml). The phases were separated, and the organic phase was passed over annydrous sodium sulfate, filtered, dried under argon, and stored at -20 C. The sample was dissolved in 200ul methanol, and centrifuged at 12,000 x g for 2 minutes to remove particulate matter prior to analysis by HPLC.

HPLC Analysis

The solvent delivery system consisted of a Beckman (Beckman Instruments, Inc., Irvine, CA) Model 322 MP programmable HPLC with CRIA integrating printer plotter. The system was equipped with a Model 153 fixed-wavelength UV (254 nm) detector, and fractions were collected directly in scintillation minivials with an LKB Model 2112 fraction collector. The reversed phase column utilized was a Beckman Ultrasphere-ODS (150 x 4.6 mm).

The column was equilibrated with 85% methanol in water, and the flow rate was 1 ml/minute throughout the analysis. A 20 µl sample was injected, and elution was initiated with a mobil phase of 85% methanol in water. After 30 seconds, the methanol concentration was increased to 100% over a period of 1.5 minutes. Aliquots of 0.2 ml were collected in each minivial and 2 ml of Instagel scintillation cocktail was added. Radioactivity was measured with a Beckman LS-9000 scintillation counter. After complete elution of the hydrocarbons, the column was re-equilibrated with 85% methanol for 8 to 10 minutes. Authentic B(a)P metabolite standards were detected by UV absorbance at 254 nm.

RESULTS

Representative cultures of human peripheral lung epithelial cells (HEL), skin epithelial cells, and liver parenchymal cells are shown in Figure 1. The lung (Figure 1A) and liver (Figure 1C) cells attach to the substratum, and they will each grow to a confluent monolayer. The skin epithelial cells (Figure 1B) have the additional capability of being able to grow in a vertically stratified layer. For our purposes, proliferating cell cultures are those that have significant areas of the substratum (usually 40-50%) not covered with cells, while non-proliferating cultures are confluent. The skin epithelial cells exhibit vertical stratification under both circumstances. The proliferative states of duplicate cultures were verified by ^{3}H-thymidine-labeling and autoradiography (data not shown).

The conversion of [G-^{3}H]B(a)P to water soluble metabolites by human epithelial cells derived from lung, skin and liver is presented in Table 1. The cells from lung and liver generated significantly greater amounts of the water soluble metabolites than the skin epithelial cells.

The ethyl acetate-soluble metabolites of B(a)P generated by proliferating HEL epithelial cells is depicted in Figure 2. The HPLC data indicates that less than 30% of the organic-soluble material is accounted for by the parent hydrocarbon B(a)P. The major portion of the radioactivity (34.3%) was localized under the B(a)P-tetrol peak. The relative distribution of ethyl acetate-soluble metabolites is summarized on line one of Table 2.

A significant difference in the HPLC metabolite profile of non-proliferating HEL epithelial cells can be seen in Figure 3. Although there is little difference in the amount of B(a)P-tetrols produced by proliferating, (Figure 2) and non-proliferating (Figure 3) HEL cells, a major polar derivative peak is predominant in the latter. In addition, a B(a)P-diol peak in Figure 3 is almost non-existent. The early eluting polar component produced by non-proliferating HEL cells is made up almost totally of B(a)P sulfate conjugates because this peak is reduced greater than 80% by treatment with arylsulfatase (data not presented). Again, a summary of relative B(a)P-metabolite production by the non-proliferating HEL cells is presented in Table 2. There is a decrease in the amounts of both B(a)P diols and phenols when compared to proliferating HEL cells, and more of the B(a)P is metabolized.

Proliferating NFS epithelial cells metabolize much less B(a)P than HEL cells (Table 2). After 24 hours, 83.9% of the ethyl acetate-extractable hydrocarbon is the parent compound B(a)P. None of the B(a)P-metabolites account for more than 4.5% of the radiolabeled material.

Non-proliferating NFS epithelial cells metabolize more than twice as much B(a)P than proliferating NFS cells (Table 2). As with HEL cells, the major increase is in the synthesis of polar derivatives. The confluent NFS culture gives rise to somewhat more B(a)P-tetrol, but there is little change in B(a)P diols and phenols.

Profiles of intracellular distribution of B(a)P-metabolites (foreskin fibroblasts) revealed that a major portion of the B(a)P remains in its parent form, (21). Extracellular oxygenated metabolites account for less than 10% of the added B(a)P after 24 hour treatment, Figure 3. B(a)P-tetrols, diols and phenols accounted for less than 1% of the remaining metabolites.

Liver parenchymal cells actively metabolize B(a)P similar to HEL epithelial cells (Table 2). Only 21.1% of the organic soluble hydrocarbon is unmetabolized B(a)P after 24 hours. Polar derivatives (26.3%) and B(a)P-tetrols (25.2%) are found in significant amounts in the medium of the confluent liver cell culture. Both levels are similar to those in the confluent HEL epithelial cell culture and higher than the NFS epithelial cell culture.

DISCUSSION

Cell proliferation is required for the fixation of the carcinogenic event. We have been able to induce neoplastic transformation of normal human fibroblasts with a variety of chemicals, including B(a)P, if the cells are in an enhanced proliferative state (2,15). However, we have not been able to transform confluent, non-proliferating human cells even if the cells are subpassaged and allowed to divide immediately after the carcinogen treatment.

Even though cell proliferation is required for chemical carcinogen-induced neoplastic transformation, most studies of B(a)P activation make use of non-proliferating cell cultures (3,4,7,11,13,14,22). It has been shown with human skin fibroblasts, that stationary (non-proliferating) cultures established by seeding cells at a low density in nutrient-deficient medium yield 10 times more oxygenated B(a)P-metabolites than proliferating cultures (4). The significance of these oxygenated B(a)P metabolites to carcinogensis is unknown. We reported previously that non-confluent skin fibroblasts transport B(a)P to the nucleus while confluent fibroblast do not (2,20). Therefore, the increased metabolism by non-proliferating cultures may be without effect.

Human epithelial cells exhibit different growth characteristics <u>in vitro</u> when compared to fibroblasts, and most human cancers are of epithelial origin (carcinomas); therefore, we felt it was of interest to examine the metabolism of B(a)P, by epithelial cells under proliferating conditions which favor transformation and under non-proliferating conditions which do not favor transformation. Epithelial cells from different primary target tissues were utilized to assess intertissue variation.

The lung and liver cell cultures produce more water soluble metabolites than do the skin cells (Table 1). The water-soluble derivatives consist mainly of detoxified conjugates of B(a)P (13,22). Considering that the epidermis is an effective physical barrier, the need for an active detoxification pathway may be less important than with lung and liver.

B(a)P diol-epoxides are considered to be the ultimate carcinogenic metabolites of B(a)P (1,23). The metabolic activation of B(a)P occurs at the P450 locus in the plasma membrane, and the B(a)P-diol-epoxides synthesized form adducts predominantly with the N^{2} moiety of guanine in nucleic acids (21-23). Such interactions with DNA are thought to be responsible for the induction of carcinogenesis.

In an aqueous environment, B(a)P-diol-epoxides are rapidly hydrolyzed to B(a)P-tetrols, so these are the most significant products one can measure in the ethyl acetate phase. Little change was observed in the generation of B(a)P-tetrols by proliferating and non-proliferating human epithelial cell cultures (Table 2). This indicates that the activation pathway is functioning under both circumstances, and that the ultimate carcinogenic B(a)P-diol-epoxides are synthesized.

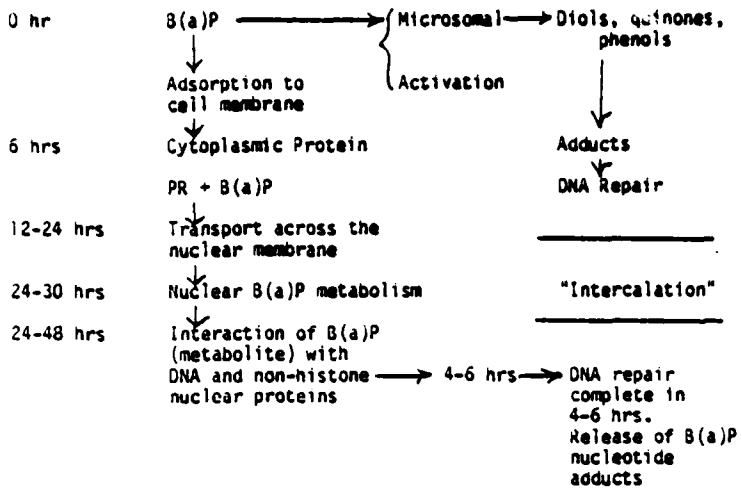
The early eluting polar B(a)P metabolite peak (Figure 3; Table 2) is composed predominantly, if not totally, of B(a)P sulfate conjugates. Extraction of these detoxification products of B(a)P phenols with ethyl acetate has been reported using a different chromatographic system (7,24). Our results for human epithelial cells are consistent with this detoxification pathway being activated in non-proliferating cells. With the HEL cells (Figure 2 and 3; Table 2), the increase in the early polar derivative(s) in the confluent culture is accompanied by a decrease in B(a)P-phenols and diols.

The results presented in this manuscript demonstrate that human lung epithelial cells and liver parenchymal cells biotransform B(a)P to a much greater extent than do human skin epithelial cells. The increased biotransformation involves both the activation and the detoxification pathways. In addition, non-proliferating epithelial cells have a greater propensity for deactivating B(a)P than do proliferating epithelial cells. This may help explain the need for cell proliferation during carcinogen exposure in order to obtain transformed cells in vitro.

Transformable fibroblast cell populations (2) produce less than 3% hydroxylated-epoxide metabolites (oxygenated forms), (21). In excess of 98% of the B(a)P remains as B(a)P.

The intracellular distribution of B(a)P in the fibroblasts appears to occur as B(a)P bound to a low molecular weight lipoprotein (21). Our present DNA adduct data (Tejwani, Jeffery and Milo, unpublished data) suggests that the ultimate major carcinogenic form in the nucleus is Benzo(a)Pyrene 7,8 diol 9,10 epoxide -I (anti)-deoxyguanosine . This adduct has been reported by others to be the major adduct excised by the error free repair system from the DNA, (25,26). In fibroblasts, activation by the microsomal P450 complex is not necessary to biotransform B(a)P to an oxygenated carcinogenic derivative, however activation must occur prior to induction of carcinogenesis. We conclude from these data that the oxygenation of the B(a)P to the carcinogenic metabolite must take place in another intracellular location, presumably the nucleus.

A proposed mechanism for the biotransformation of B(a)P in fibroblasts followed by the induction of a carcinogenic event is presented here.



Similar studies are in progress with human epithelial cells from target tissues to describe B(a)P activation and induction of a carcinogenic event.

Table 1

Partitioning of Radioactivity After Incubation of Human Epithelial Cells with [G-^{3}H]B(a)P for 24 Hours

Cells	Percent Organic-soluble	Water-soluble
HEL		
Proliferating	60	40
Non-proliferating	52	48
NFS		
Proliferating	77	23
Non-proliferating	81	19
Liver		
Non-proliferating	42	58

TABLE 2

B(a)P Metabolites Produced by Human Epithelial Cells After Incubation with [G-^{3}H]B(a)P for 24 Hours[a].

Cells	Polar Derivatives[b]	B(a)P Tetrols	B(a)P Diols	Unidentified Metabolite[c]	B(a)P Phenols	B(a)P
HEL						
Proliferating	---	34.3	13.7	9.5	12.2	26.4
Non-proliferating	32.1	27.5	2.0	6.9	3.8	9.2
NFS						
Proliferating	1.9	4.5	1.2	3.4	2.1	83.9
Non-Proliferating	17.0	9.4	1.8	3.0	1.8	60.5
Liver						
Non-proliferating	26.3	25.2	3.5	11.6	3.2	21.1

[a]The values are expressed as percent of total radioactivity, and they represent the integrated values under the HPLC peaks.

[b]Treatment with aryl sulfatase removes greater than 80% of this component indicating it is predominantly a sulfate conjugate of B(a)P.

[c]B(a)P-6,12-dione and 6-hydroxymethyl-B(a)P co-elute at this position.

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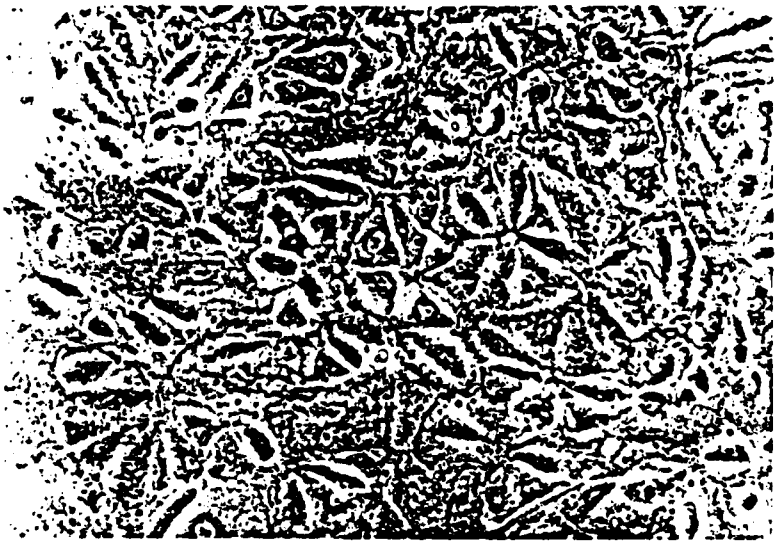
REFERENCES

1. Milo, G., Blakeslee, J., Yohn, D., and DiPaolo, J. Biochemical activation of AHH activity, cellular distribution of polynuclear hydrocarbon metabolites and DNA damage by PNH products in human cells in vitro. (1979), Cancer Res. 38, 1638-1644.
2. Ekelman, K., and Milo, G. Cellular uptake, transport, and macromolecular binding of benzo(a)pyrene and 7,12 dimethylbenz(a)anthracene by human cells in vitro. (1978), Cancer Res. 38, 3026-3032.
3. Baird, W., and Diamond, L. Metabolism and DNA binding of polycyclic aromatic hydrocarbons by human diploid fibroblasts. (1978), Int. J. Cancer 22, 189-1975.
4. Rudiger, H., Marxen, J., Kohl, E.V., Melderis, H., and Wichert, P. Metabolism and formation of DNA adducts of Benzo(a)pyrene in human diploid fibroblasts. (1979), Cancer Res. 39, 1083-1088.
5. Jeffrey, A., Jennette, K., Goldstein, S., Weinstein, I., Harvery, B., Kasai, H., Miura, K. and Nakanisht, K. Benzo(a)pyrene tetrahydrodiol derivative found in vivo: Structure of benzo(a)pyrene tetrahydrodiol epoxideguanosine adduct. (1976), J. Am. Chem. Soc. 98, 5714-5715.
6. Kesen, J., Jerina, D., Yagi, H., and Cerutti, P. In Vitro reaction of radioactive 7, 8-dihydroxy-9,10 epoxy-7,8,9,10-tetra-hydrobenzo(a)pyrene and 7,8-dihydroxy-98, 108 epoxy-7,8,9,10-tetra-hydrobenzo(a)pyrene with DNA. (1977), Biochem. Biophys. Res. Comm. 74, 939-940.
7. Cohen, G., Haws, S., Moore, B., and Bridges, J. Benzo(a)pyrene-3-ol hydrogen sulphate a major ethyl acetate-extractable metabolite of benzo(a)pyrene in human: hamster and rat lung cultures. (1976), Biochem. Pharmacol. 25, 2561-2570.
8. Diamond, L. etabolism of polycyclic hydrocarbons in mammalian cell culture. (1971), Int. J. Cancer 8, 451-457.
9. Nebert, D.W., and Gelboin, H.V. Substrate-inducible microsomal aryl hydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. (1968), J. Biol. Chem. 243, 6242-6249.
10. Huberman, E., Sachs, L., Yang, S.K., and Gelboin, H.V. Identification of mutagenic metabolites of benzo(a)pyrene in mammalian cells. (1976), Proc. Nat. Acad. Sci. U.S.A. 73, 607-611.
11. Fox, C., Selkirk, J., Price, F., Croy, R.G., Sanford, K., and Cottler-Fox, M.L. Metabolism of benzo(a)pyrene by human epithelial cells in vitro. (1975), Cancer Res. 35, 2551-2557.
12. Trewyn, R., Douglas, W., and Milo, G. Resolution of benzo(a)pyrene metabolites generated by human lung epithelial cells. *(1980), Carcinogensis, (submitted).*
13. Yamasaki, H., Huberman, E., and Sachs, L. Metabolism of the carcinogenic hydrocarbon benzo(a)pyrene in human fibroblasts and epithelial cells. II Differences in metabolism to water-soluble products and aryl hydrocarbon activity. (1977), Int. J. Cancer 19, 378-382.
14. Yang, S., Gelboin, H., Trump, B., Autrup, H. and Harris, C. Metabolic activation of benzo(a)pyrene and binding to DNA in cultured human bronchus. (1977), Cancer Res. 37, 1210-1215.
15. Milo, G., and DiPaolo, J. Neoplastic transformation of human diploid cells in vitro after chemical carcinogen treatment. (1978), Nature 275, 130-132.
16. Riegner, D., McMichael, T., Berno, J., and Milo, G. Processing of human tissue to establish primary cultures in vitro. (1976), Tissue Culture Association Lab Manual 2, 273-276.
17. Noyes, I., Milo, G., and Cunningham, C. Establishment of proliferating human epithelial cells in vitro from cell suspensions of neonatal foreskin. (1980), Tissue Culture Association Lab Manual 5, 1173-1176.
18. Milo, G., Ackerman, A., and Noyes, I. Growth and ultrastructural characterization of proliferating keratinocytes in vitro without added extrinsic factors. (1980), In Vitro 12, 20-30.
19. Schaeffer, W., and Kessler, D. Human liver cells in culture. (1980), In Methods in Cell Biology, (ed. D. Prescott) Academic Press N.Y., (in press).
20. Tejwani, R., Nesnow, S., and Milo, G. Analysis of intracellular distribution and binding of benzo(a)pyrene in human diploid fibroblasts. (1980), Cancer Letters, 10, 57-65.
21. Tejwani, R., Trewyn, R., and Milo, G. Benzo(a)pyrene metabolism by transformable human skin fibroblasts. (1980), Fed. Amer. Soc. for Exp. Biol., Proc. 39, 2570.
22. Moore, C., and Schwartz, A. Inverse correlation between species lifespan and capacity of cultured fibroblasts to convert benzo(a)pyrene to water-soluble metabolites. (1978), Exptl. Cell Res. 116, 359-364.
23. Levin, W., Wood, A.W., Waslocki, P.G., Change, R.L., Kapitulnik, J., Mah, H.D., Yagi, H., Jerina, D.M., and Conney, A.H. Mutagenicity and carcinogenicity of benzo(a)pyrene and benzo(a)pyrene derivatives. Gelboin, H.V. and Tso, P.O.P. (eds) (1978), In Polycyclic Hydrocarbons and Cancer, Vol. 1, Academic Press, New York, pp. 189-202.
24. Cohen, G., Mehta, R., and Meredith-Brown, M. Large interindividual variations in metabolism of *Benzo(a)pyrene by peripheral lung tissue from lung cancer patients.* (1979), Int. J. Cancer 24, 129-133.
25. Cerutti, P.A., Sessions, F., Hariharan, P., and Lusby, A. Repair of DNA damage induced by benzo(a)pyrene diolepoxides I and II in human alveolar tumor cells. (1978), Cancer Res. 38, 2118-2124.
26. Yang, L., Maher, V., McCormick, J. Excision repair of the N^2 guanine adduct of "anti" 7,8-Diol 9,10-epoxide of Benzo(a)pyrene by human cells is error-free. Fed. Amer. Soc. of Exp. Biol. Proc. 39, 1738.

Figure 1

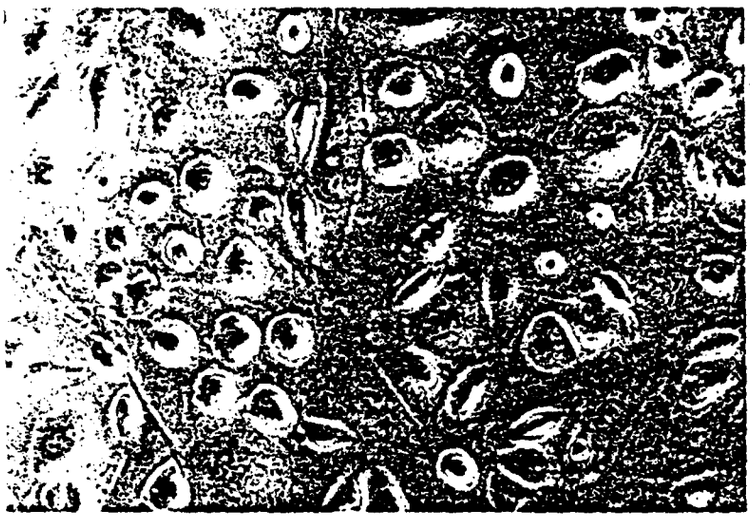
A. Human peripheral embryonic lung cells at passage 1 at saturation density state of growth, 100 X.



B. Human foreskin epidermal cells at a saturation density state of growth at passage 1, 100 X.

Figure 1

C. Human embryonic liver at passage 2 at a saturation density state of growth, 160 X.



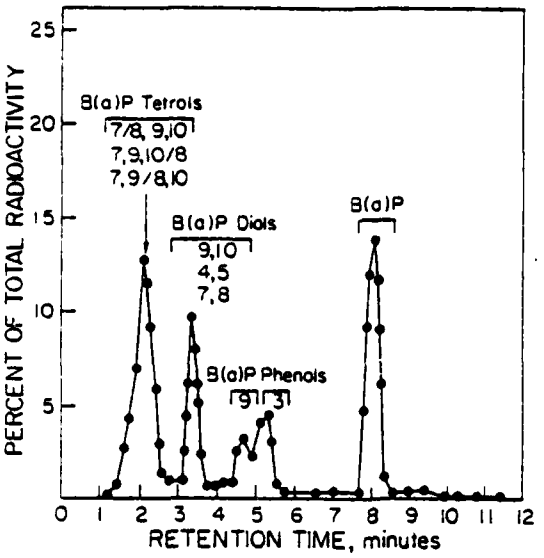


Figure 2. Metabolite profile of B(a)P produced by proliferating HEL cells. The activity is expressed as a percent of the total radioactivity of B(a)P plotted as a function of the retention time on the HPLC column .

Figure 3. Metabolite profile of B(a)P produced by HEL cells at a confluent density. The activity is expressed as outlined in Figure 2, legend.

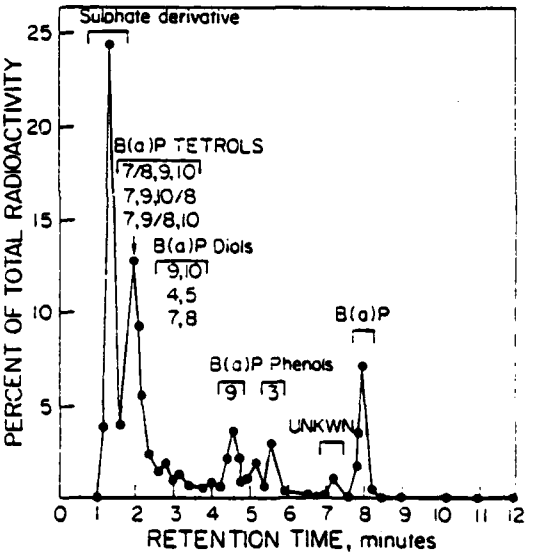
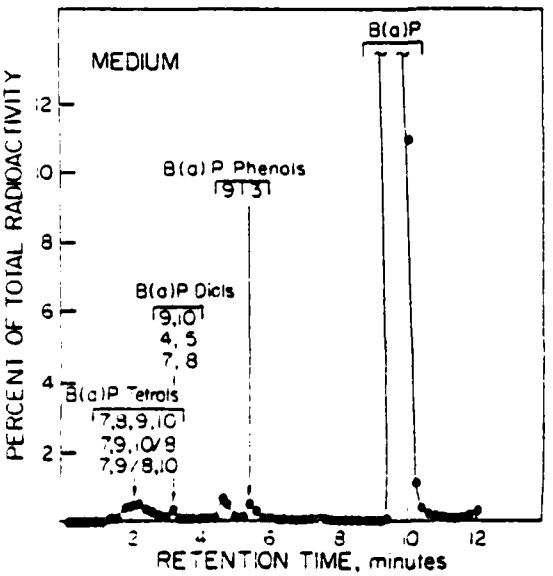


Figure 4. Metabolite profile of B(a)P produced by proliferating ,transformable foreskin fibroblasts. The activity is expressed as outlined in Figure 2, legend.



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CHARACTERISTICS OF BENZO[*a*]PYRENE AND A-RING REDUCED 7,12-DIMETHYL BENZ[*a*]ANTHRACENE INDUCED NEOPLASTIC TRANSFORMATION OF HUMAN CELLS IN VITRO*

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SUMMARY

The polynuclear aromatic hydrocarbons (PAH) benzo[*a*]pyrene (BP) and the A-ring reduced analogue of 7,12-dimethylbenz[*a*]anthracene (DMBA), 1,2,3,4-tetrahydro-7,12-dimethylbenz[*a*]anthracene (TH-DMBA) are carcinogenic to human cells. The unsaturated PAH, DMBA exhibits no carcinogenic activity on human cells as measured by growth in soft agar. The TH-DMBA and BP treated cells exhibit a colony frequency in soft agar of 84 and 86, respectively. These anchorage independent cells, when seeded on the chick embryonic skin (CES) organ cultures, are invasive and form a fibrosarcoma. It is highly unlikely that TH-DMBA, which does not contain an aromatic A-ring, can undergo metabolism in human cells in culture to form a bay region 3,4-dihydrodiol-1,2-epoxide. These results suggest that an alternate mechanism for the induction of carcinogenesis is appropriate to explain the absence of bay region diol-epoxide metabolite as the ultimate form of the carcinogen in TH-DMBA induced carcinogenesis in human diploid cells.

INTRODUCTION

Metabolic conversion of PAH such as BP and DMBA has been shown to precede an expression of their toxic, mutagenic or carcinogenic activities [1,2,5,6,17,21]. Our previous studies with BP have indicated that this car-

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cinogen can induce neoplastic transformation in proliferating human skin fibroblast cells when added during the S phase of the cell cycle [4, Milo et al., unpublished data]. Although DMBA can induce a carcinogenic event in rodent cells in culture [12], this PAH does not induce such an event in proliferating human skin fibroblast cells in culture [4].

Previous results from our laboratory have indicated that BP is taken up and initially bound to a cytoplasmic lipoprotein complex present in human skin fibroblast cells before being transported to the nucleus [4,19, Tejwani, unpublished data]. DMBA, on the other hand, is randomly dispersed throughout these cells and is not bound to the cytoplasmic lipoprotein complex [4, Tejwani, unpublished data]. These differences may in part explain the induction of neoplastic transformation in the normal human skin fibroblast cells by BP and not by DMBA.

The bay region diol-epoxide of DMBA, the 3,4-dihydrodiol-1,2-epoxide, has been proposed to be the major metabolite responsible for the mutagenic and carcinogenic activities of this PAH in rodent cells [3,18]. Consistent with this proposal, DMBA exhibits mutagenicity in the Ames assay only in the presence of a microsomal activation system [7].

The observation that the PAH TH-DMBA was mutagenic in the absence or presence of a microsomal activation system, using 3 strains (TA1537, TA98, TA100) of *Salmonella typhimurium* [7], provided impetus to study this A-ring reduced analogue for carcinogenic activity, using human cells. Since TH-DMBA cannot be expected to yield a bay region diol-epoxide unless it was first oxidized (aromatized) to DMBA, this A-ring reduced analogue should serve as a useful probe for investigating alternate mechanisms of transformation.

In our laboratory, we have defined several indices of human fibroblast cell transformation in response to a variety of chemical carcinogens. These include morphological changes, extended life span, growth in culture conditions toxic for untreated normal cells, increase in lectin agglutinability and alteration in cellular prostaglandin levels [10]. The anchorage-independent growth of transformed cells in soft agar has been the most consistent and reliable indicator of tumor production in athymic nude mice and on the CES organ culture system.

In this report, data are presented on the carcinogenicity of TH-DMBA in human neonatal foreskin (HNF) fibroblast cells in culture.

MATERIALS AND METHODS

Chemicals

DMBA was purchased from Eastman Chemical Company and BP was received from the National Cancer Institute Repository. TH-DMBA was synthesized by a method previously described [7,20]. The hydrocarbon was purified on a Spherisorb ODS 5 μm column (4.5 mm × 25 cm) using a linear gradient of 25—100% methanol for 1 h. All solvents were of reagent or analytical grade.

Cell cultures and treatment of cells with PAH

Primary HNF cell cultures were established as described previously [14]. Randomly proliferating cell populations, derived from human foreskin tissues dispersed with collagenase, were passaged in complete growth medium (CM) composed of Eagle's minimum essential medium: 25 mM Hepes buffer (pH 7.2) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 50 μg/ml gentocin, 0.2% sodium bicarbonate and 10% fetal bovine serum (FBS) in a 4% CO_2-enriched air atmosphere at 37°C. HNF cells passaged at a 1 : 4 split ratio reached confluency in 4 days.

Preconfluent (70—80%) logarithmically growing HNF cell populations between population doubling (PDL) 4 to 5 were blocked at the G_1/S phase of the cell cycle by feeding the cultures with non-proliferating Dulbecco's modified Eagle's medium supplemented with 50 μg/ml gentocin, 1 mM sodium pyruvate and 10% dialyzed FBS [8—10]. Twenty four hours after seeding, when the mitotic index was 0.1% to 0%, the non-proliferating medium was removed and the cell cultures were re-fed with CM supplemented with 0.5 U/ml insulin. Thirty four hours after seeding, the synchronized cell cultures were treated with 3.2 μg/ml of BP, 1 μg/ml of DMBA or 0.5 μg/ml of TH-DMBA in acetone. Cell cultures to which an equal volume of acetone was added served as controls. The carcinogen was allowed to remain in contact with the cells during the S phase of the cell cycle which was 8.2 h long [8—10].

Selection of transformed cell populations

Forty eight hours after seeding, the cell cultures were passaged at a 1 : 2 split ratio into CM supplemented with 2× essential vitamins, 8× non-essential amino acids and 20% FBS (selection medium). The cell populations were serially passaged at 1 : 10 split ratio into the selection medium [8—10]. After 16 PDL, the cell populations were seeded into soft agar.

Anchorage independent growth

The treated and control cell populations were trypsinised and seeded at 50,000 cells/25 cm^2 well in 2 ml of 0.33% soft agar and Dulbecco's LoCal medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 50 μg/ml gentocin, 0.2% sodium bicarbonate, 1× essential vitamins, 1× essential amino acids and 20% FBS. The cells were layered over 5 ml of a 2% agar base prepared in RPMI 1629 medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 50 μg/ml gentocin, 0.2% sodium bicarbonate, 1× essential vitamins, 1× essential amino acids and 20% FBS [8—10]. The soft agar cultures were incubated at 37°C in a 4% CO_2-enriched air atmosphere. Three weeks later, the colonies were removed with a tuberculin syringe (20 gauge needle) and seeded into the selection medium. After attachment and sufficient growth, the cell cultures were trypsinized and re-seeded in order to evenly distribute the cells from the monolayer colonies.

The cell cultures were passaged once at a 1 : 4 split ratio and then seeded on the CES to evaluate neoplasia.

Neoplastic transformation

The cell populations at PDL 36 were seeded on the CES organ cultures prepared from 9 to 18-day-old fertilized eggs [Milo et al., unpublished data]. The CES were layered onto an agar base containing 10 parts of 1% Agar in Earle's balanced salt solution, 4 parts of chick embryo extract and 4 parts of FBS.

Then, cells (10^5) from treated and control cultures were suspended in 0.025 ml CM supplemented with 20% FBS and seeded on the CES. Three days later, the skins were removed and fixed in Bouin's solution. Transverse sections (5 μm) were stained with hematoxylin and eosin and examined for the presence of invasive features.

RESULTS

Chemical carcinogen induced neoplastic transformation of HNF cells in culture was carried out by the procedure of Milo and DiPaolo [8,9]. Randomly proliferating low passage cell populations were seeded into amino acid deficient medium to block them at the G_1/S phase of the cell cycle. The cell populations were released from the block in presence of a growth promoter. Ten hours later, in the S phase of the cell cycle, the cell cultures were treated with BP, DMBA or TH-DMBA. Cell populations transformed by BP, DMBA or TH-DMBA were serially passaged for 16 PDL

TABLE 1

CHARACTERIZATION OF TRANSFORMED HUMAN FORESKIN FIBROBLAST CELLS THROUGH ANCHORAGE INDEPENDENT STAGE AND NEOPLASTIC STAGE OF CARCINOGENESIS

Compound	Concentration (μg/ml)[a]	Evidence of colony formation in soft agar[b]	Incidence of tumor formation in CES[c]
BP	3.2	86	1/1[d]
DMBA	1	0	N.D.
TH-DMBA	0.5	84	1/1

[a] The concentration of each of these compounds was appropriately selected from results of previous work [4].

[b] Four wells (25 cm²/well) were seeded with 50,000 cells/well in 0.33% soft agar supplemented with growth medium, over a 2% agar base [8—10]. The values for colony formation reported here were the number of colonies formed per 10^4 cells seeded into soft agar [8—10].

[c] These values reported here were for 4 CES seeded with 10^5 cells/CES and evaluated 3 days later. Normal untreated cells did not invade the tissue.

[d] BP treated cells were also evaluated for tumor formation in nude mice [4]. The treated cells were positive and formed tumors.

and seeded into soft agar to evaluate anchorage independent growth. BP (3.2 μg/ml) treated cells exhibited anchorage independent growth with a frequency of colony formation of 86/10^5 cells (Table 1). Although DMBA (1 μg/ml) did not transform HNF cells in culture as measured by anchorage independent growth, cells transformed by TH-DMBA (0.5 μg/ml) grew to spherical colonies 10—12 days after seeding on soft agar (Fig. 1), with a frequency of colony formation of 84/10^5 cells (Table 1).

The BP and TH-DMBA treated cell populations exhibiting anchorage independent growth were seeded onto the CES to evaluate neoplasia. These transformed cell populations were invasive on the CES and produced fibrosarcomas (Fig. 2). The tumors were designated as fibrosarcomas because of the presence of mitotic figures in the tumor tissue.

Following growth in soft agar and re-seeding into monolayer cultures, BP treated cells were also evaluated for neoplasia in nude mice. Six-week-old mice received 5 × 10^6 cells subcutaneously. Four weeks later, the tumors were excised and submitted to histopathology for evaluation. The tumors produced were identified as fibrosarcomas.

Fig. 1. Randomly proliferating HNF cell populations blocked in the G_1 phase of the cell cycle were released from the block and subsequently treated with TH-DMBA. The cell populations were serially passaged for 16 PDL and seeded at 50,000 cells/25 cm^2 well into soft agar as described under Materials and Methods. The soft agar cultures were incubated at 37°C in a 4% CO_2-enriched air atmosphere for 3 weeks. Each colony contained 50—300 cells, 14 days following seeding into soft agar.

A

B

Fig. 2. The BP or TH-DMBA treated cell populations exhibiting anchorage independent growth were removed and serially passaged. Then, 10^5 cells suspended in 0.025 ml CM supplemented with 20% FBS were seeded on the CES [Milo et al., unpublished data]. Three days later, the CES were fixed in Bouin's solution, stained with hematoxylin and eosin and microscopically examined. (A) represents normal untreated fibroblast cells seeded on the CES at 160× magnification. (B) represents TH-DMBA treated cell populations growing on the CES at 160× magnification.

DISCUSSION

The PAH TH-DMBA was mutagenic in the Ames assay both with and without metabolic activation in 3 strains of *S. typhimurium* [7]. In fact, this A-ring reduced analogue was more mutagenic in the absence of microsomal activation using the plasmid deficient strain TA1537 [7]. TA1538 and the missense tester strain TA1535, which are relatively insensitive to DMBA mutagenesis were not mutated by TH-DMBA [7]. Nonetheless, DMBA required metabolic activation by addition of the S9 microsomal fraction in order to elicit mutagenicity in strains TA1537, TA98 and TA100 [7], whereas TH-DMBA required no such metabolic activation. TH-DMBA, in the absence of the S9 fraction had mutagenic properties similar to metabolically activated DMBA. This is a marked departure from other PAH which require an activating system or chemical modification to a reactive species to effect mutagenesis in the Ames assay. Thus, even 3,4-dihydrodiol-7-methylbenz[*a*]-anthracene was non-mutagenic in the strain TA98, when cofactors required for the microsomal activation system were omitted [11].

Previous results have indicated that when HNF cells are treated with BP, a major portion of the PAH is bound to a cytoplasmic lipoprotein complex and is subsequently transported to the nucleus as the parent compound [4,19, Tejwani, unpublished data]. HPLC analysis of the BP metabolites covalently bound to DNA has indicated a low level of BP metabolism taking place in these cells, with the formation of a small amount of the BP-7,8-diol-9,10-epoxide-I-deoxyguanosine adduct [Tejwani, unpublished data]. In the present study, BP also induced neoplastic transformation in the HNF cells and the treated cell populations exhibited anchorage independent growth with a frequency of colony formation of $86/10^5$ cells seeded in soft agar. These transformed cell populations were invasive on the CES and produced a fibrosarcoma.

Although DMBA did not transform HNF cells in culture as measured by anchorage independent growth, it was interesting to observe that TH-DMBA, the analogue of DMBA completely reduced in the bay region, could induce neoplastic transformation in these cells and was as potent as BP, at 1/6 the dose of BP used. This pronounced activity of TH-DMBA is similar to what would be expected in our system if we were assessing an ultimate carcinogen.

Previous reports have indicated that 1,2,3,4-tetrahydro-7-methylbenz[*a*]-anthracene, a 12-desmethyl analogue of TH-DMBA, is non-carcinogenic [16]. It is known that the 12-methyl function in DMBA provides sufficient steric interaction with the C_1-carbon—hydrogen of the A-ring to stabilize a ketone function at position 5 owing to decreased planarity of the tetracyclic system [13,14]. In TH-DMBA the C_{12}—C_1 interaction freezes the ring A in a half-chair conformation with C_2 slightly and C_3 markedly out of plane with the aromatic anthracene system. The speculative A-ring triol of DMBA, possibly arising by reaction of DNA or other macromolecular nucleophile with the proposed bay region 3,4-dihydrodiol-1,2-epoxide, is expected to have a similar conformation.

These observations question the necessity for metabolic activation in the case of TH-DMBA as a prerequisite to macromolecular binding and subsequent transformation in human cells. Work is in progress employing radiolabeled TH-DMBA to further assess this phenomenon.

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REFERENCES

1 Autrup, H., Harris, C.C., Trump, B.F. and Jeffrey, A.M. (1978) Metabolism of benzo-(a)pyrene and identification of the major benzo(a)pyrene-DNA adducts in cultured human colon. Cancer Res., 38, 3689—3696.
2 Chou, M.W. and Yang, S.K. (1978) Identification of four trans-3,4-dihydrodiol metabolites of 7,12-dimethylbenz(a)anthracene and their *in vitro* DNA-binding activities upon further metabolism. Proc. Natl. Acad. Sci. U.S.A., 75, 5466—5470.
3 Dipple, A. and Nebzydoski, J.A. (1978) Evidence for the involvement of a diol-epoxide in the binding of 7,12-dimethylbenz[a]anthracene to DNA in cells in culture Chem.-Biol. Interact., 20, 17—26.
4 Ekelman, K. and Milo, G.E. (1978) Cellular uptake, transport and macromolecular binding of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene by human cells *in vitro*. Cancer Res., 38, 3026—3032.
5 Feldman, G., Remsen, J., Wang, W. and Cerutti, P. *(1980) Formation and excision of* covalent DNA adducts of benzo(a)pyrene 4,5-epoxide I in human lung cells A549. Biochemistry, 19, 1095—1101.
6 Huberman, E., Chou, M.W. and Yang, S.K. (1979) Identification of 7,12-dimethylbenz-(a)anthracene metabolites that lead to mutagenesis in mammalian cells. Proc. Natl. Acad. Sci. U.S.A., 76, 862—866.
7 Inbasekaran, M.N., Witiak, D.T., Barone, K. and Loper, J.C. (1980) Synthesis and mutagenicity of A-ring reduced analogues of 7,12-dimethylbenz(a)anthracene. J. Med. Chem., 23, 278—281.
8 Milo, G.E. and DiPaolo, J. (1978) Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment. Nature (London), 275, 130—132.
9 Milo, G.E. and DiPaolo, J.A. (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis, Int. J. Cancer, 26, 805—812.
10 Milo, G.E., Oldham, J., Zimmerman, R., Hatch, G. and Weisbrode, S. (1981) Characterization of human cells transformed by chemical and physical carcinogens *in vitro*. In Vitro, in press.
11 Malaveille, C., Bartsch, H., Tierney, B., Grover, P.L. and Sims, P. (1978) Microsome-mediated mutagenicities of the dihydrodiols of 7,12-dimethylbenz(a)anthracene: high mutagenic activity of the 3,4-dihydrodiol. Biochem. Biophys. Res. Commun., 83, 1468—1473.
12 Marquardt, H., Baker, S., Tierney, B., Grover, P.L. and Sims, P. (1979) Comparison of mutagenesis and malignant transformation by dihydrodiols from benz(a)anthracene and 7,12-dimethylbenz(a)anthracene. Br. J. Cancer, 39, 540—547.
13 Newman, M.S. and Olson, D.R. (1974) A new hypothesis concerning the reactive

species in carcinogenesis by 7,12-dimethylbenz(a)anthracene. The 5-hydroxy-7,12-dimethylbenz(a)anthracene-7,12-dimethylbenz(a)anthracene-5(6H)-one equilibrium. J. Am. Chem. Soc., 96, 6207—6208.

14 Newman, M.S. and Cunico, R.F. (1972) The synthesis of 5-hydroxymethyl-, 5-acetoxymethyl- and 5-methylmercapto-7,12-dimethylbenz(a)anthracenes and of 5,7,12-trimethylbenz(a)anthracene. J. Med. Chem., 15, 323—325.

15 Riegner, D.T., Berno, M. and Milo, G.E. (1976) Processing of human tissue to establish primary cultures *in vitro*. Tissue Culture Association Lab. Manual, 2, 273—276.

16 Shear, M.J. (1938) Studies in carcinogenesis of methyl derivatives of 1:2-benzanthracene. Am. J. Cancer, 33, 499—537.

17 Sims, P., Grover, P.L., Swaisland, A., Pal, K. and Hewer, A. (1974) Metabolic activations of benzo(a)pyrene proceeds by a diol-epoxide. Nature (London), 252, 326—328.

18 Slaga, T.J., Gleason, G.L., DiGiovanni, J., Sukumaran, K.B. and Harvey, R.G. (1979) Potent tumor-initiating activity of the 3,4-dihydrodiol of 7,12-dimethylbenz(a)-anthracene in mouse skin. Cancer Res., 39, 1934—1936.

19 Tejwani, R., Nesnow, S. and Milo, G.E. (1980) Analysis of intracellular distribution and binding of benzo(a)pyrene in human diploid fibroblasts. Cancer Letters, 10, 57—65.

20 Witiak, D.T., Inbasekaran, M.N., Cazer, F.D., Daniel, F.B. and Hart, R.W. (1979) A novel synthesis of 1,2-dihydro-7,12-dimethylbenz(a)anthracene: a key intermediate leading to the synthesis of proposed A-ring ultimate carcinogenic metabolites of DMBA. Abstract, 177th National Meeting of the American Chemical Society, Honolulu, Hawaii.

21 Yang, L.L., Maher, V.M. and McCormick, J.J. (1980) Excision repair of the N^2-guanine adduct of "anti" 7,8-diol-9,10-epoxide of benzo(a)pyrene by human cells is error free. Fed. Proc., 39, 1738.

IN VITRO
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FELINE SARCOMA VIRUS INDUCED IN VITRO PROGRESSION FROM PREMALIGNANT TO NEOPLASTIC TRANSFORMATION OF HUMAN DIPLOID CELLS

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SUMMARY

Human diploid cells morphologically transformed by feline sarcoma virus were serially propagated under selective cell culture conditions. When injected into nude mice prior to passage in soft agar (0.35%), morphologically transformed cells did not produce tumors. However, when propagated under selective cell culture conditions, transformed cells grew in soft agar and, when injected subcutaneously into the subcapsular region of the nu/nu mice, produced neoplastic nodules histopathologically interpreted as fibromas. Karyological examination of cell populations grown out from the tumors confirmed that the tumors were composed of human cells. Examination of electron micrographs of the excised tumor tissue revealed the presence of budding virus particles. Tumor cells isolated from nude mice and morphologically transformed cells both contained the feline oncornavirus-associated cell membrane antigen. It was concluded that expression of feline oncornavirus-associated cell membrane antigen is associated with an early stage of feline retrovirus-induced carcinogenesis, namely focus formation. In addition, it was shown that FeLV-FeSV can induce morphological transformation in human cells in vitro and that there is a requirement for the cells to passage through soft agar before subsequent tumor formation (neoplastic transformation) can be demonstrated.

Key words: feline sarcoma virus; neoplastic transformation; human diploid cells.

INTRODUCTION

Feline retroviruses have been reported to transform cells of numerous animal species including hamsters (1-4), cats (5-7), dogs (2,4,8,9), pigs (2,5), sheep (10), monkeys (11), and humans (4,7,9,10, 12-15) morphologically. However, rat and mouse cells are refractory (16), along with WI-38 cells (17), to feline retrovirus transformation and thus there are no reports about neoplastic transformation, i.e., the ability to produce tumors with the in vitro transformed cells in an appropriate animal host.

Although the role of feline retrovirus in spontaneous neoplastic diseases of various heterologous animal hosts is not known, the feline sarcoma virus (FeLV/FeSV) induces fibrosarcomas in cats (5,6,18) and other species. The oncogenic properties of FeLV FeSV in humans are not known.

The objectives of this study were to determine the optimal in vitro conditions of transformation of human diploid cells by FeLV FeSV and the oncogenic potential of these transformed cells in nu/nu mice.

MATERIALS AND METHODS

Primary NFS cultures. Primary human cell cultures (NFS) established from foreskin tissue as previously described (19), were maintained on Eagle's minimal essential medium (MEM), Hanks' buffered salt solution (HBSS), 25 m*M*

HEPES buffer at pH 7.2 (GIBCO, Grand Island, NY), 1 m*M* sodium pyruvate, 2 m*M* glutamine, 50 μg Gentamycin/ml (Schering Diagnostics, Port Reading, NJ), 0.2% sodium bicarbonate, and 10% fetal bovine serum (FBS) (Biofluids, Inc., Rockville, MD).

Preparation of stocks of Snyder-Theilen FeSV. Stocks of ST-FeSV were prepared as previously described (20). Feline embryo cells at 50 to 70% confluency were treated with DEAE-dextran (40 μg/ml) in L-15 medium (GIBCO) at room temperature for 20 min. Following removal of the DEAE-dextran, a virus inoculum in L-15 medium supplemented with 5% FBS was added to the cell sheet for 2 hr. The cell sheet was then refed with L-15 medium + 15% FBS and incubated for 7 to 10 days at 37° C in a 4% CO_2-enriched atmosphere. The cells were harvested by scraping and subjected to two cycles of rapid freezing in dry ice: 95% ethanol and thawing in a 37° C water bath. The cell suspensions were centrifuged at 350 ×*g* for 10 min; the supernatant solution was filtered through a 0.45-μm Millipore filter and stored in 0.5-ml aliquots at −70° C.

Selection of "pure" populations of FeSV-infected human cells. Preconfluent cell populations at population doubling (PDL) 4 to 14 (1) were seeded at 0.5 to 1.0 × 10^4 cells/cm^2 in MEM + 10% FBS. After each split at a 1:4 split ratio, the PDL were increased by 2. After 24 hr, the cells were inoculated with 1:250 dilution of FeSV, which had a titer of 6.1 × 10^6 focus forming units (ffu)/ml. The protocol for inoculation was identical to that used on the human diploid cell cultures except that MEM growth medium was used in place of L-15. Ten days later, the cultures, which contained 10 foci/cm^2 for a 20-cm^2 plate, were passaged 1:4. Those areas in the confluent culture containing hyperrefractile cells that stained densely with hematoxylin were identified as foci (12). The cultures attained confluency in 5 days and were serially passaged thereafter 1:10 until "pure" populations were attained that contain 100% feline oncornavirus cell membrane antigen (FOCMA)-positive cell populations. These infected cell populations required 8 to 9 days to reach saturation density.

Effects of different growth media on proliferation of FeSV-infected cells. Cells were seeded at 1:10 dilutions into different growth media supplemented with 10% FBS. Cell proliferation was then monitored in either McCoy's 5A, MEM-Mg^{++}, MEM (GIBCO) or EBM LoCal (Biolabs, Northbrook, IL). McCoy's 5A medium was used previously for the growth of feline leukemia virus-transformed (FL-74) cells in suspension culture (20); MEM-Ca^{++} - Mg^{++} and EBM LoCal were selected because these elements have previously been found to alter Adeno-12-induced focus formation of hamster embryo cells (21) and to alter susceptibility and refractoriness of Yaba tumor pox virus-induced focus formation (22).

Release of infectious virus from FeSV-infected human cells. Pure populations of FeSV-infected human cells growing on either EBM LoCal, McCoy's 5A, or MEM media were assayed for the release of infectious virus. Twenty-four hours after seeding at PDL 20, or as the cells stopped proliferating, aliquots of the supernatant solutions were removed and filtered through a 0.22-μm Swinnex Millipore filter, then diluted and used as inoculum. Ten days later, the infected plates were fixed in 10% formalin and stained with hematoxylin and eosin. Finally, the foci were enumerated. These foci contained cells that were morphologically distinct from the normal cells (*see above*) and are hereafter referred to as morphologically transformed cells.

Passage of morphologically transformed cells through soft agar. Soft agar was used as a suspension medium for the growth of morphologically FeSV-transformed cells. A 2% agar base, RPMI 1629 medium (GIBCO) was supplemented with 20% FBS. Transformed cells were harvested from the supernatant solution and resuspended in EBM LoCal medium + 20% FBS, 1% essential amino acids, 1% essential vitamins, 0.35% agar, 1 m*M* sodium pyruvate, 2 m*M* glutamine, 0.2% sodium bicarbonate, and 50 μg/ml Gentamycin. Two milliliters of this cell suspension were seeded at 1-2 × 10^5 cells/ml over the agar base plates. These media were used because the morphologically transformed cells grow more optimally in these media. We have tried RHMI 1640, MEM, BME, *et cetera* with less success.

FOCMA detection. An indirect immunofluorescence test for FOCMA (23) was performed on the morphologically transformed cells. Proliferating transformed cells from either monolayers or soft agar were harvested by centrifugation at 650 ×*g* for 7 min at a cell density of 0.5 to 1.0 × 10^6. The reference primary reagent (cat serum) used in this study was from a FOCMA antibody-positive cat that was persistently viremic. This agent was shown to be specific for FOCMA, since absorption of the serum with intact and ether-disrupted (FeLV (10^{11} purified particles/ml of serum) did not decrease antibody

titers (24). In addition, this reagent produced membrane fluorescence on FeSV-infected human neonatal foreskin cells, but not on uninfected human foreskin cells (*unpublished data*).

Histopathology and electron microscopy. Tumors of FeSV-infected cells and FeSV-infected cell populations prepared from boluses growing in soft agar were prepared for histopathology and electron microscopy.

Athymic nude (nu/nu) mice, which were backcrossed 5 or 10 times and obtained from Sprague-Dawley, Madison, WI, were selected for evaluation of the neoplastic potential of FeSV-transformed human cells. Preconfluent cultures of FeSV-transformed cells were prepared for injection by scraping with a rubber policeman; they were then pelleted by centrifugation at 540 ×*g* for 7 min. The pellet was resuspended in fresh MEM and recentrifuged. After resuspension in MEM + 0.5% agar, 0.53 to 1.0 × 10^7 cells were injected subcutaneously into athymic nude (*nu/nu*) mice that had been irradiated 3 to 4 days previously with 450 rad ^{137}Cs γ-rays. The nodules that developed at the site of inoculation were excised after 6 weeks' growth, fixed in 3% glutaraldehyde–0.1 *M* cacodylate buffer at pH 7.4, and then prepared for histopathology and electron microscopy. In addition, FeSV-infected proliferating populations isolated from boluses obtained from soft agar were scraped from the substratum of the flasks, pelletized by centrifugation at 650 ×*g* for 7 min, and fixed in 3% glutaraldehyde–0.1 *M* cacodylate buffer at pH 7.4 for examination under an electron microscope.

Karotype analysis of excised tumors. Tumors from 0.8 to 1.2 cm in length were identified at the site of injection after 6 weeks. These tumors were surgically removed from the nude mice and cell suspensions were made as described elsewhere (19). Hyperimmune antiserum prepared against nude mouse skin cells was added to the culture of 500,000 tumor cells in a 75-cm^2 flask at 0.6 ml/15 ml of growth medium. The medium containing the antiserum was renewed every 24 hr. Seventy-two hours later, the proliferating cells were refed with 5 μg/ml of colcemide (GIBCO) in 10 ml of growth medium and incubated for 3 hr at 37° C. The medium was decanted after 3 hr and the cell monolayer was rinsed with warm PBS-Ca^{++} - Mg^{++}. Following their removal with 10 ml of trypsin-versene solution (40:1), the cells were recovered by centrifugation at 650 ×*g*. The cell pellet was fixed in cold methanol-glacial acetic acid (3:1). A suspension was dropped onto a glass slide, dried, and stained with prefiltered 5% Giemsa solution.

FIG. 1. This photograph shows proliferating FeSV-transformed human cells. A supernatant cell suspension from a culture at PDL 17 containing 50 to 80% transformed cells was harvested by centrifugation at 650 ×*g* for 7 min and the cell pellet was resuspended and seeded in MEM + 10% FBS. Other nonattached cells remain rounded up and floating in the medium. ×28.

RESULTS

Selection of pure populations of FeSV-infected cells. At 5 to 10 PDL after selective subculturing of the virus-infected human cells was started, it became impossible to distinguish individual foci in the culture because of the increased number of infected cells in the population. Many hyperrefractile, round FeSV-infected cells were released into the growth medium. Populations of 1 to 2×10^6 cells/ml were harvested from this "breeder" culture. Two methods of harvesting these free-floating cells resulted in the selection of two cell types. Centrifugation and resuspension in fresh MEM + 10% FBS gave rise to cells that attached to the substratum and exhibited a variety of cellular morphologies (Fig. 1). Direct transfer of the old media and cells into a flask produced a seeding suspension; some of these cells attached to the substratum, whereas others began to grow into large boluses in suspension; they varied in size and contained from 25 to 200 cells (Fig. 2). Both harvesting methods gave rise to cultures containing pleomorphic cellular and colony morphology.

Growth characteristics in different culture media. We have tried many recipes for media. Table 1 lists their ability to support the growth of the transformed cells. MEM-Mg^{++} + 10% FBS, McCoy's 5A + 10% FBS, and McCoy's 5A supplemented with 5 μg/ml spermidine + 10% FBS supported cell growth for less than 3 PDL. McCoy's 5A + 5 μg/ml uridine; 10% FBS supported growth for 3 to 5 PDL.

FeSV-infected cells subpassaged 1:10 into EBM LoCal medium plus 10% FBS proliferated for 0 PDL; however, further subpassaging resulted in cell lysis. Cultures that were subpassaged on MEM-HEPES died after 42 to 46 PDL.

Release of infectious virus. The supernatant solutions were individually assayed for infectious virus (see Materials and Methods) after 20 PDL or when the cells ceased proliferating and lysed.

The data in Table 2 indicate that only MEM and EBM LoCal supported the production and release of infectious virus. There were 350 times

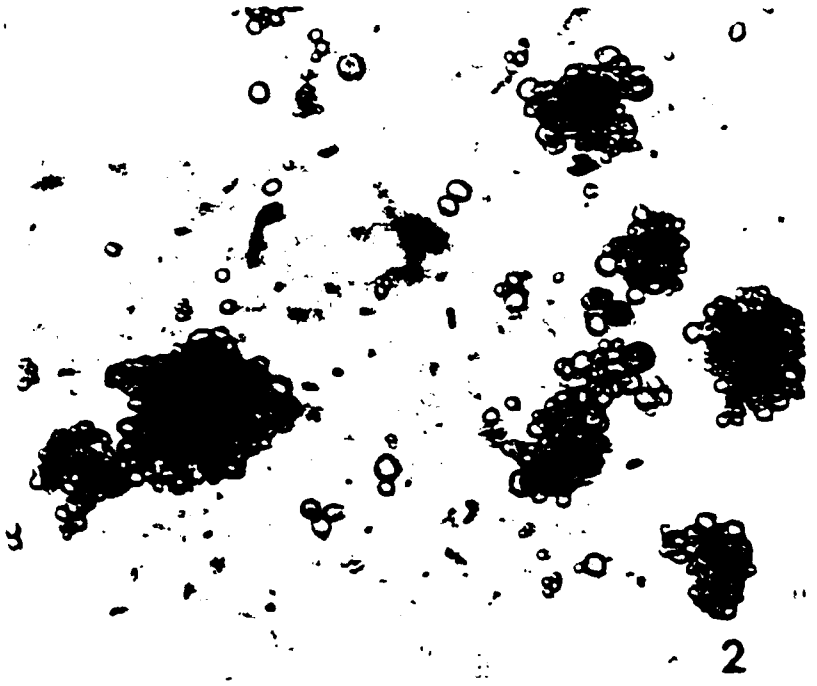

FIG. 2. A cell suspension recovered without centrifugation from the overlying growth medium of FeSV-transformed human fibroid cells PDL 16 was seeded directly into a flask without replenishing the growth media. Ten days later actively dividing boluses of cells were observed. ×40.

TABLE 1

GROWTH OF FeSV-TRANSFORMED CELLS IN DIFFERENT GROWTH MEDIA

Growth Medium	PDL
1. MEM + 25 m*M* HEPES	46
2. MEM - Mg^{++}-Ca^{++}	<3
3. McCoy's 5A	0
4. McCoy's 5A + 5 μg/ml spermidine	<2
5. McCoy's 5A + 5 μg/ml uridine	4
6. EBM LoCal	7

The proliferative characteristics of a pure FeSV-transformed human cell population were measured as PDL on different types of growth media. Other experiments on other populations for PDL 1 through 6, not reported here, supported these data. In most cases the mean PDL did not exceed one sigma SD for values reported here. PDL as referenced in the text is: 1 serial subpassage at 1:2 split ratio at 95% absolute plating efficiency. One population doubling (PDL) is that cell population that was serially passaged 1:4 at confluency. If the cells were serially passaged at 1:4 they had proceeded through PDL.

more focus forming units released from cells grown on MEM than from cultures grown on LoCal. This suggests that virus production and release, like cell proliferation, is dependent on the specific cell culture medium used. Calcium has also been shown to alter virus-induced cell morphology and to produce biochemical changes in other virus-cell systems (15,25). Cell populations grown in McCoy's 5A-supplemented growth medium died after a short time. There was no release of detectable infectious units.

Growth in soft agar. Single cell clones were obtained by cloning single cell suspensions in agar to evaluate the infected cells for their ability to grow in soft agar. Free-floating cells were seeded at 1 to 2×10^4 cells/ml into soft agar after 16 to 20 PDL and round compact colonies observed 10 to 14 days later containing 50 to 100 cells/bolus (Fig. 3). The frequency of bolus formation was 2.5 to 5×10^{-4}.

FOCMA expression on FeSV-infected cells. Randomly proliferating cells were assayed for the presence of FOCMA by indirect immunofluorescence (26). Figure 4, *A* is a light micrograph of several infected cells. Figure 4, *B* illustrates the fluorescent pattern of FOCMA on the same cells. The pattern is typically patchy. We observed a similar pattern of FOCMA fluorescence by FL-74 cells grown in spinner flasks (20). To date, all transformed cells treated with fluorescein isothiocyanate tag control cat serum have been negative.

Growth of FeSV-transformed human cells in nude mice. To evaluate the neoplastic potential of these FeSV-transformed proliferating human cells, 0.5 to 1.0×10^7 cells were injected into nude mice. Twenty-four hours later, the bleb at the injection site regressed. After 5 to 30 days, palpable nodules were evident. They increased in size to 0.8 to 1.4 cm over a 4-week period at the end of which the nodules were excised and prepared for histopathology.

Histopathology of tumors from nude mice. Histologically, the nodules from both mice were well encapsulated, sharply demarcated cellular masses (Fig. 5). The neoplastic cells were ovoid to spindle shaped and contained a single vesiculated nucleus usually with a prominent nucleolus. The cytoplasmic margins were frequently indistinct and some cells appeared to form syncytia. An application of the Massons trichrome stain showed the eosinophilic fibrillar intercellular material to be collagen. A basophilic (hematoxylin) mucinlike intercellular material was admixed with the collagen fibers and stained with Alcian Blue. This revealed the presence of acid mucopolysaccharides

TABLE 2

MEASUREMENT OF FOCUS FORMING UNITS ON APPROPRIATE MONOLAYER CULTURES

Growth Medium	ffu/75-cm² flask[a]
1. MEM + 25 m*M* HEPES	$1.9 \times 10^7 \pm 1.4 \times 10^6$
2. MEM-Mg^{++}-Ca^{++}[b]	—
3. McCoy's 5A[b]	—
4. McCoy's 5A + 5 μg/ml spermidine[b]	—
5. McCoy's 5A + 5 μg/ml uridine[b]	—
6. EBM LoCal	$5.5 \times 10^6 \pm 1.1 \times 10^6$

[a] Dashes (—) mean that ffu were not detected when supernatant solutions were assayed on appropriate human monolayer cultures (19) (morphological transformation) after the cell populations ceased to proliferate and lysed (Table 1).

[b] Cells cultured in these media exhibited limited proliferative capability and ceased proliferating prior to the virus assay period.

These figures show the results of a focus-forming assay of FeSV-transformed cells cultured in different growth media. NFS cells were originally infected with a 1:250 dilution of FeSV from a pool with a titer of 6.1×10^6 ffu/ml. The virus assay period was determined to be at least 20 PDL at 1:10 split ratios after virus infection for cells grown on MEM-HEPES medium. This time period was selected in order to ensure that only pure populations of virus-transformed cells were assayed. Focus-forming units/75-cm² flask were determined for 1×10^6 to 2.0×10^6 cells per experiment. These data represent the results for n of 4 for MEM at PDL-20 and n of 3 for LoCal at PDL 7. They are presented here as mean values ± 1 sigma SD.

(Fig. 6). Mitotic figures were rare, the mass was well vascularized, and the neoplastic cells were located immediately adjacent to the vessels. The mass was interpreted to be a fibroma.

FIG. 3. A suspension of free-floating viable cells in the overlying growth medium from a FeSV-transformed culture at PDL-12 was harvested by centrifugation at 650 ×g for 7 min. Cell populations at 1-2 × 10^5 cells/ml were seeded into 0.5% soft agar containing LoCal growth medium. Ten days after seeding, colonies of cells were observed. ×65.

FIG. 4. Proliferating FeSV-transformed cells, 0.5-1.0 × 10^6, were harvested by centrifugation at 650 ×g and incubated with FOCMA antibody reference cat serum, obtained from an animal with a regressing sarcoma, for 30 min. Cells were washed free of unbound cat globulins with three consecutive rinses of Hanks' balanced salt solution and incubated with a 1:20 dilution of goat anticat gammaglobulin (Sylvania, Inc., Milburn, NJ), which had been conjugated to fluorescein isothiocyanate. *A*. Light micrograph of FeSV-transformed cells; *B*. UV-fluorescence micrograph of the same field. ×197.

Under electron microscopic examination, cells in culture and cells from the nodules removed from mice were both found to contain viruslike particles (Fig. 7).

FIG. 5. A well-encapsulated subcutaneous nodule in an nu/nu mouse. The cells are individually aligned (*arrow*) or arranged in syncytial sheet(s). ×8.

FIG. 6. Electron micrograph of cell from subcutaneous nodule in *nu/nu* mouse. The interstitium contains flocculent electron-dense material (*arrowhead*) and numerous fibrils (*arrow*) containing the characteristic 640-A banding of collagen. ×30,000.

FIG. 7. Virus particles (*arrows*) budding from cell membrane of fibroblast in culture prior to injection into nu/nu mouse. ×22,400.

TABLE 3

MODALITY OF HUMAN CHROMOSOMES OF CELLS PREPARED FROM TUMOR CELLS

PDL[a]	Modal No.	Range
10	45	43–47
10	43	42–45
5	46	45–47
7	46	45–48
6	45	44–46

[a] These PDL represent proliferation of population of FOCMA-positive cells seeded from the tumor. The range represents the distribution of chromosomes seen on a single slide.

The data presented here represent our attempt to count the numbers of human chromosomes found in the cells that produced tumors in the nude mice. The tumors were removed, seeded in vitro, and at subsequent PDL, evaluated.

Distribution of chromosome number in tumor material. Tumors excised from nude mice were grown in vitro as described previously. In no case, of the metaphase spreads from five different tumors evaluated, was there significant deviation from the diploid number of chromosomes.

DISCUSSION

Replication of FeLV-FeSV in human cells and subsequent focus formation were demonstrated in this study, confirming previous reports (9,10,15, 26–29). The data obtained in this study, however, suggest that focus formation (i.e., morphological transformation) (2) represents only a transitional stage in the neoplastic transformation process. It is interesting to note that Azokar and Essex (17) did not observe morphologically transformed cells when WI-38 were infected with FeSV. However, if we add Fungizone or penicillin and streptomycin to the cultures much in the same manner they did, no foci were observed. This study showed that the separation of morphologically transformed from nontransformed normal cells and growth as well as passage in soft agar were prerequisites for the demonstration of neoplastic properties by the transformed cells. The separation was accomplished by culturing and passaging the cells in a low-calcium supplement growth medium for 2 PDL to 5 PDL in MEM-HEPES EBM-LoCal medium. The resultant transformed cell populations grew in suspension, whereas populations that contained normal-appearing cells did not. Selective culturing in suspension of these cells in EBM-LoCal medium followed by subculturing in MEM 5 PDL later resulted in the cells reattaching to the substratum. It was necessary to serially subpassage these cells for an additional 16 to 20 PDL in 1 × MEM-HEPES growth medium before they would grow in soft agar. The morphologically transformed cells, when passaged through soft agar, formed colonies of 50 to 100 cells/bolus at a frequency of 2.5 to 5 × 10^{-4}/25 cm^2. These cells, isolated from soft agar and grown in a selective medium, were injected into 6-week-old nude mice to evaluate the oncogenic potential. Nutritional requirements and time in culture appear to determine growth and expression of the neoplastically transformed cells. Selective nutritional requirements have also been shown for other FeSV-transformed cell systems such as transformed feline producer cells, which grow optimally on McCoy's 5A (22); or Ad-12-transformed hamster cells, which require a low-Ca^{+2} growth medium (21). The transformed human cells that also grew on EBM-LoCal medium did not grow on MEM minus Mg^{+2} - Ca^{+2}.

Tumors produced in the nude mice (0.8 to 1.4 cm in size) were found to contain collagen and were interpreted histopathologically to be fibromas, not fibrosarcomas (25). Removal of the tumor was followed by growth of the cells in culture; subsequent karyological examination indicated that the tumor cells were of human origin. Examination of electron micrographs of excised tumors and proliferating cells from in vitro populations of morphologically transformed cells, and examination of cells passaged through agar, revealed that both types of cells were shedding virus particles. Assay of the morphologically transformed cell populations (30) before and after growth in soft agar revealed that FOCMA was present at both stages in the transformation sequence. These results suggest that FOCMA expression is associated with the early events in the transformation process. There appears to be a program or staging process that must occur in FeVL/FeSV-transformed human cells before they will produce tumors when injected into a suitable host. The results reported here appear to be similar to the multistage process of chemical carcinogen-induced transformation (25). FOCMA and virus expression appear to be associated with early stages of morphological transformation, whereas growth in soft agar is associated with a later transitional stage. A high correlation exists between growth in soft agar and tumor formation. It was noted that although virus-infected cells would not produce tumors prior to their growth in soft agar, after they were

passaged through the soft agar they would produce tumors. Again, this suggests that growth in agar acted as a selection process to permit the proliferation of cells that can produce tumors when inoculated into a suitable host. Therefore, we have shown that FeSV neoplastically transformed cells are capable of producing tumors in nude mice.

The release of the infectious virus from human cells that form tumors or grow in soft agar raises the question of possible horizontal transmission. FeLV and FeLV/FeSV have been shown to be horizontally transmissible among cats (30), and FeLV, under laboratory conditions, was horizontally transmissible to dogs (15). A serological survey indicated that no antibody to FeLV occurred in hundreds of individuals exposed to viremic cats (12,31). However, Jacquemin et al. (32) reported that purified human IgG from patients with chronic myelogenous leukemia specifically neutralized reverse transcriptase isolated from FeLV.

REFERENCES

1. Gilden, R. V.; Lee, Y. K.; Long, C. Rescue of the murine sarcoma virus genome from nonproducer cells by the RD-114 type-C virus. Int. J. Cancer 10: 458–462; 1972.
2. Lee, K. M. Comments on feline leukemia virus. J. Am. Vet. Med. Assoc. 158: 1037–1039; 1971.
3. Monti-Bragadin, C.; Ulrich, K. Rescue of the genome of the defective murine sarcoma virus from a non-producer hamster tumor cell line, PM-1, with murine and feline leukemia viruses as helpers. Int. J. Cancer 9: 383–392; 1972.
4. Sarma, P. S.; Huebner, R. J.; Baskar, J. R.; Vernon, L.; Gilden, R. V. Feline leukemia and sarcoma viruses: Susceptibility of human cells to infection. Science 168: 1098–1100; 1970.
5. Jarrett, O.; Laird, H. M.; Hay, D.; Crighton, G. W. Replications of cat leukemia virus in cell cultures. Nature 219: 521–522; 1968.
6. Rickard, C. G.; Post, J. E.; deNoronha, D.; Barr, L. M. A transmissible virus-induced lymphocytic leukemia of the cat. J. Natl. Cancer Inst. 42: 987–1014; 1969.
7. Theilen, G. H.; Kawakami, T. G.; Rush, J. D.; Munn, R. J. Replication of cat leukemia virus in cell suspension cultures. Nature 222: 589–590; 1969.
8. Essex, M.; Kawakami, T. G.; Kurata, K. Continuous long-term replication of feline leukemia virus (FeLV) in an established canine cell culture (MDCK) (36129). Proc. Soc. Exp. Biol. Med. 139: 295–299; 1972.
9. Jarrett, O.; Laird, H. M.; Hay, D. Growth of feline leukemia virus in human, canine and porcine cells. Dutcher, R. H. ed. Comparative leukemia research. Basel: Karger; 1970: 387–392.
10. Chang, R. S.; Golden, H. D.; Harrold, B. Propagation in human cells of a filterable agent from the ST-feline sarcoma. J. Virol. 6: 599–603; 1970.
11. Deinhardt, F.; Wolfe, L. G.; Theilen, G. H.; Snyder, S. P. ST-feline fibrosarcoma virus: Induction of tumors in Marmoset monkeys. Science 167: 881–882; 1970.
12. Schaller, J. P.; Milo, G. E.; Blakeslee, J. R.; Olsen, R. G.; Yohn, D. S. Influence of glucocorticoid, estrogen and androgen hormones on transformation of human cells *in vitro* by feline sarcoma virus. Cancer Res. 36: 1980–1987; 1976.
13. Chan, E. W.; Schiop-Stansly, P. E.; O'Connor, T E. Mammalian sarcoma-leukemia viruses. 1. Infection of feline, bovine, and human cell cultures with Snyder-Theilen feline sarcoma virus. J. Natl. Cancer Inst. 52: 473–478; 1974.
14. Essex, M.; Klein, G.; Deinhardt, F.; Wolfe, L.; Hardy, W., Jr.; Theilen, G.; Pearson, L. Induction of the feline oncornavirus-associated cell membrane antigen in human cells. Nature (New Biol.) 238: 187–189; 1972.
15. McAllister, R. M.; Nicolson, M.; Gardner, M. B.; Rongey, R. W.; Rasheed, S.; Sarma, P. S.; Huebner, R. J.; Hatanaka, M.; Oroszlan, S.; Gilden, R. V.; Kabigtin, A.; Vernon, K. RD-114 virus compared with feline and murine type C viruses released from RD cells. Nature (New Biol.) 242: 75–78; 1973.
16. Laird, H. M.; Harrett, O.; Whalley, J. M. Electron microscopy of early interactions between feline leukemia virus and cells in tissue culture. Dutcher, R. M.; Chieco-Bianchi, L. eds. *Unifying concepts of leukemia*. 1973.
17. Azokar, L.; Essex, M. Susceptibility of human cell lines to feline leukemia virus and feline sarcoma virus. J. Natl. Cancer Inst. 63: 1179–1184; 1979.
18. Theilen, G. H.; Hall, J. G.; Pendry, A.; Glover, D. J.; Reeves, B. R. Tumors induced in sheep by injecting cells transformed *in vitro* with feline sarcoma virus. Transplantation 17: 152–156; 1974.
19. Riegner, D. A.; McMichael, T.; Berno, J. C.; Milo, G. E. Processing of human tissue to establish primary cultures *in vitro*. TCA Manual 2: 273–276; 1976.
20. Olsen, R. G.; Milo, G. E.; Schaller, J. P.; Mathes, L. E.; Heding, L.; Yohn, D. S. Influence of culture conditions on growth of FL-74 cells and *feline oncornavirus cell membrane* associated antigen production. In Vitro 12: 37–43; 1976.
21. Milo, G. E.; Schaller, J. P.; Yohn, D. S. Hormonal modification of adenovirus transformation of hamster cells *in vitro*. Cancer Res. 32: 2338–2347; 1972.
22. Milo, G. E.; Yohn, D. S. Alterations of enzymes associated with plasma membranes and cellular organelles during infection of CV-1 cells with Yaba tumor poxvirus. Cancer Res. 35: 199–206; 1975.
23. Essex, M.; Klein, G.; Snyder, S. P.; Harrold, J. G. Antibody to feline oncornavirus-associated cell membrane antigen in neonatal cats. Int. J. Cancer 8: 384–390; 1971.

24. Wolff, L. H.; Mathes, L. E.; Olsen, R. G. Recovery of soluble oncornavirus-associated cell membrane antigen from large volumes of tissue culture fluids. J. Immunol. Methods. In press.

25. Milo, G. E.; DiPaolo, J. A. Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment. Nature 275: 130–132; 1978.

26. Hampar, B.; Kelloff, G. J.; Martos, L. M.; Oroszlan, S.; Gilden, R. V.; Walker, J. L. Replication of murine and feline RNA-containing C-type viruses in human lymphoblastoid cells. Nature 228: 857–859; 1970.

27. Rickard, C. G.; Post, J. E.; deNoronha, F.; Barr, L. M. Interspecies infection by feline leukemia virus: Serial cell-free transmission in dogs of malignant lymphomas induced by feline leukemia virus. Dutcher, R. M.; Chieco-Bianchi, L. eds. Unifying concepts of leukemia. Basel: Karger; 1973: 102–112.

28. Jarrett, O.; Laird, H. M.; Hay, D. Growth of feline leukemia virus in human cells. Nature 224: 1208–1209; 1969.

29. Krakower, J. M.; Aaronson, S. A. Seroepidemiologic assessment of feline leukemia virus infection risk for man. Nature 273: 463–464; 1978.

30. Essex, M.; Cotter, S. M.; Stephenson, J. R.; Aaronson, S. A.; Hardy, W. D., Jr. Leukemia, lymphoma and fibrosarcoma of cats as models for similar disease of man. Hiatt, Watson and Winsten eds. Origins of human cancer. Cold Spring Harbor Conferences on Cell Proliferation. 1977: 1197–1214.

31. Schneider, R.; Riggs, J. L. A serological survey of veterinarians for antibody to feline leukemia virus. J. Am. Vet. Med. Assoc. 162: 217–219; 1973.

32. Jacquenin, P. C.; Saxinger, C.; Gallo, R. C. Surface antibodies of human myelogenous leukemia leukocytes reactive with specific type-C virus reverse transcriptase. Nature 276: 290–230; 1978.

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ULTRAVIOLET RADIATION-INDUCED NEOPLASTIC TRANSFORMATION OF NORMAL HUMAN CELLS, IN VITRO*

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SUMMARY

Human foreskin cell cultures in scheduled DNA synthesis (S phase) of the cell cycle were exposed to UV irradiation at a dose of 10 $J \cdot m^{-2}$ in the presence of insulin. These treated cell populations, when selectively passaged in a high amino acid supplemented complete growth medium (CM) after 20 Dulbecco's phosphate buffered saline (pH 6.8) (PDL), were able to be grown in soft agar. These treated cell populations were also grown in 1% serum supplemented growth medium and at 41°C in 10% serum supplemented growth medium. Cell populations 4—5 PDL after treatment exhibited altered colony morphology and altered lectin agglutination profiles but would not grow in soft agar. These events appeared to be associated with the early stages in the expression phase of the transformed phenotype. After 20 PDL, we observed that these cells would grow in soft agar at a frequency of 20 colonies/10^5 cells seeded in soft agar. The cell populations derived from these colonies, when propagated and injected into the nude mice, formed myxofibromas at the injection sites rather than the type of tumor (fibrosarcoma) previously described for chemical carcinogen-induced neoplasms.

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Abbreviations: CM, complete growth medium; FBS, fetal bovine serum; MEM, minimum essential medium; NFS, neonatal foreskins; PDL, Dulbecco's phosphate buffered saline (pH 6.8); RCE, relative colony-forming efficiency; S phase, scheduled DNA synthesis.

INTRODUCTION

Carcinogen-induced transformation of human cells in vitro by chemicals or irradiation has been difficult. Recently, however, neoplastic transformation of human cells by chemicals [1] and viruses [2] has been achieved. In addition, Sutherland [3], using UV treatment of human cells, damaged the DNA and transformed the cell populations to an anchorage independent state of growth. Multiple treatments at subtoxic doses of UV were necessary to damage the DNA and induce transformation. Other studies with refractory human cells have indicated that carcinogens can induce unscheduled DNA synthesis [4] or repair synthesis [5]. However, these reports do not attempt to correlate damage to DNA with expression of carcinogenesis. Instead, Heflich [6] correlated removal of chemical carcinogen and induced damage to DNA with cytotoxicity. We have expanded on our preliminary report of UV-induced neoplastic transformation of human cells [7] and present data on the reproducibility of the process. We also present data on the interrelationship between anchorage-independent growth and tumor growth as the normal cells pass through a retro-differentiated sequence from induction to neoplasia.

MATERIALS AND METHODS

In order to have observed reproducible transformation of human cells exposed to UV, it was necessary, first, to use low passaged cell populations (PDL 1—5) and, second, to complete a cell survival curve prior to the selection of an appropriate treatment dose.

Cell cultures

Primary cell cultures were obtained from neonatal foreskins (NFS) as previously described [8]. They were maintained on CM, viz., minimum essential medium (MEM): 25 mM Hepes, Gibco, Grand Island, New York) at pH 7.2, supplemented with 10% fetal bovine serum (FBS), (Rehatuin, Kankakee, IL), 1 mM sodium pyruvate, 2 mM glutamine, 0.2% sodium bicarbonate and 50 μg/ml gentomycin, in an atmosphere of 4.0% CO_2-enriched air at 37°C.

Survival studies of irradiated cells

Immediately after UV treatment or 40 h later, cell survival was measured by either dye exclusion [9], colony-forming ability [10] or incorporation of [^{3}H]thymidine into cellular DNA [9]. After seeding, cultures were irradiated with a 15 W Germicidal Electric lamp (15GT8) at a fluence rate of 1.2 $J \cdot m^{-2} \cdot sec^{-1}$. The fluence rate was measured by a Blak-Ray UV meter (UV Products, International, San Gabriel, CA). To measure colony forming capability of the treated cell population, one thousand cells were seeded in 25 cm^2 wells (Falcon Plastics, Oxnard, CA) and fed with CM supplemented with 20% FBS. These cultures were incubated at 37°C in a

4% CO_2-enriched air atmosphere for 9 days, fixed in phosphate-3% formalin stained with hematoxylin-eosin and enumerated under 23×. If the effect of the treatment on cell survival was to be measured by dye exclusion, approx. 5000 cells · cm^{-2} in a 25-cm^2 dish were trypsinized at the conclusion of the UV treatment and counted in a hemocytometer [9]. Inhibition of the incorporation of CH_3-[^{3}H]thymidine (S.A. 54.0 Ci/mmol) into the DNA of UV-treated cells was measured by sampling the cell population from 0—48 h following initiation of UV treatment [8]. At 1-h intervals, 3 coverslip cultures were removed, fixed in 3 : 1 methanol/acetic acid solution, acid washed in 1.0 N HCl and air dried. Two coverslips were transferred to scintillation vials and incubated overnight with 0.5 ml of NCS tissue solubilizer, then neutralized with 1 N NaOH. Insta-Gel (10 ml) (Beechman Inst., Palo Alto, CA) was added to each vial and the radioactivity counted in a Beckman scintillation counter.

Induction

The scheme of induction and selection of the transformed cells will be presented along with the chronology of the events as they occurred (Fig. 1).

An irradiation dose permitting 50% of the cells to survive was optimal for the formation of transformants in each cell population studied [1]. Preconfluent, logarithmically growing cultures at low (3—8) PDL were synchronized by placing them at a density of 5000 cells · cm^{-2} into Dulbecco's Modified Eagle's Medium (Biolabs, Northbrook, IL) at pH 7.2, supplemented with 10% dialyzed FBS but lacking arginine and glutamine. After 24 h, the amino acid deficient medium was replaced with CM containing 0.5 U/ml of insulin. Then hours later, when the cell population was entering S phase of the cell cycle [1], the growth medium was removed, the cultures were washed twice with PDL, irradiated with 5.0 J · m^{-2} of UV and refed with CM containing 0.5 U/ml insulin. Upon completion of the treatment, the cells were allowed to proceed through S phase, which required 8.2 h, and one mitosis. They were then split 1 : 2, then 1 : 10, into MEM supplemented with 10% FBS plus 8× non-essential amino acids (Microbiological Assoc., Rockville, MD) (8× growth medium). The 8× growth medium inhibited growth of cells that exhibited a normal phenotype [1] while allowing proliferation of treated-altered cells.

The treated cell population at this time was identified as entering the early stage of the carcinogenesis process. During this stage of expression it is imperative that no part of culture remain in a saturation density growth state for more than 16 h prior to passaging the culture.

Early

Growth of cells at 41°C and in medium with reduced serum concentration. Cell populations, following treatment with UV, were seeded (5000 cm^{-2}) in CM and serially passaged at 1 : 4 on a 4-day schedule and maintained at 41°C in a 4% CO_2-enriched air environment. Cell populations from companion cultures were seeded at 5000 cells · cm^{-2} in CM supplemented with

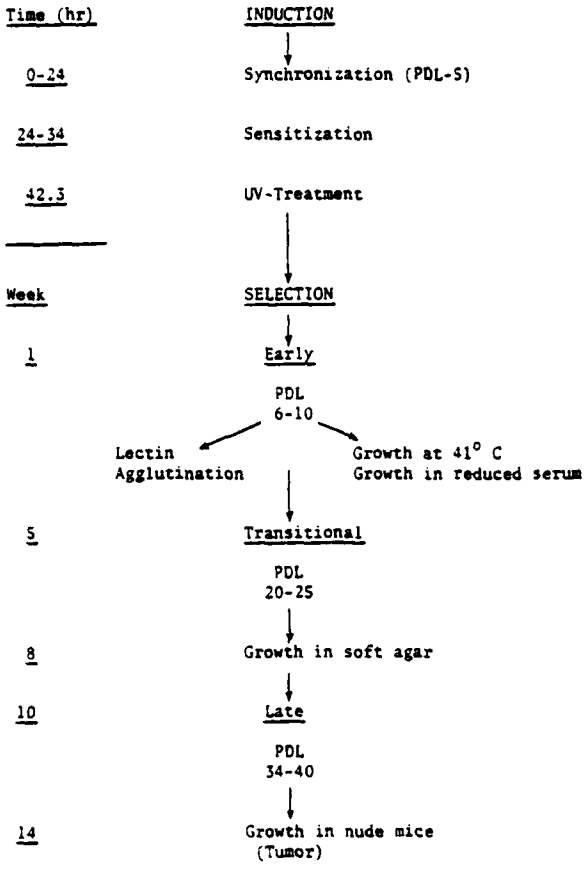

Fig. 1. The scheme followed here is a graphic representation of the sequence originating with the induction (treatment in early S) through tumor formation. Each critical stage is represented by the PDL (population doubling) reached at each stage of the process. Transition from colony formation is soft agar prior to growth in the nude mouse was achieved following isolation and seeding of colonies in 75 cm² flasks to produce 5 × 10⁶ cells of inoculum for each mouse.

1% FBS and maintained also in a 4% CO_2-enriched air environment at 37°C.

Lectin agglutination. Treated and controlled populations at a 70—90% confluent density of proliferating cells were removed from the substratum of the flask by the action of 0.05% trypsin [8]. Following centrifugation at 650 × *g*, the pellet was resuspended in PBS (pH 6.8) and recentrifuged twice. The final cell suspension was left on wet ice at a cell density of 10^6 cells · ml^{-1}. Wheat germ agglutinin prepared in PBS was added to the

wells of microtiter plates (0.025 ml). To this, 0.025 ml of a suspension of 180 000 cells in PBS was added to each well and incubated at 21°C for 10 min. The wells were then examined immediately.

Altered colony formation. Fibroblast cultures were treated as described in the induction protocol. Four PDL, following irradiation of the treated cells, were seeded into 60-mm diameter wells at a 1000 cells/well into 8X growth medium. After incubation at 37°C in a 4% CO_2 humidified enriched air atmosphere for 9 days, the cells were fixed with 3% PBS buffered formalin (pH 7.2), stained with hematoxylin and examined for colonies with abnormal colony morphology. The abnormal colonies were comprised of polygonal-small cells that overlapped into a criss-cross disoriented growth pattern. This growth pattern was in contrast to the normal large fusiform shaped fibroblasts growing in a parallel-whorling orientation.

Transitional

Soft agar. After serial passage of the UV-treated cells for 20 PDL in 8X amino acid supplemented CM, 50 000 cells were seeded in 2 ml of 0.33% agar supplemented with modified Dulbecco'c Lo-Cal medium (Biolabs, Northbrook, IL). These cells were then overlaid on a 2 ml 2% agar base supplemented with RPMI 1629 growth medium enriched with 20% FBS. Colonies formed after 14—17 days of incubation in a 4% CO_2-enriched air environment at 37°C [1]. These cultures were refed every 7 days with 0.5 ml of Lo-Cal medium-supplemented agar. Since a close correlation has been shown between the growth of chemically transformed human cells in soft agar and their neoplastic potential [1], growth of the UV-treated cells in 6-week-old male athymic nu/nu mice from a BABL/c background (Sprague—Dawley, Madison, WI) were chosen as a suitable assay system to determine the neoplastic potential of the culture derived from UV-treated cells.

Late

Growth in nude mouse. Cells were harvested by trypsinization and resuspended in Dulbecco's EMB LoCal (Biolabs, Northbrook, IL), supplemented with 20% FBS and 1X essential amino acids and 0.35% agar. Six-week-old nude mice, which have been irradiated 3—4 days previously with 450 rad ^{137}Cs-rays, were injected subcutaneously with a cell mass of 0.5×10^7 UV-treated cells. Nodules became evident at the site of inoculation 14—21 days following injection and continued increasing in size up to 4—6 weeks. The blebs formed at the injection site regressed in 48—72 h. The tumors were excised between 4 and 6 weeks and submitted for histopathological examination.

RESULTS

Toxic response

In the early stages of our experiments following the treatment with UV,

we measured the toxic effect the treatment had on the UV-insulin treated cells by counting the total number of viable cells at 12 h (Fig. 2) after initiating the UV treatment or at 40 h after initiating treatment (Fig. 3). In addition, recovery of cell division from the toxic effects of irradiation, monitored by [^{3}H]thymidine incorporation into DNA and by cell counts, began at 44—48 h following the completion of the treatment. The response patterns of irradiated cultures previously not exposed to insulin exhibited a typical shoulder on the survival curve followed by a logarithmic decline at doses greater than 10 J · m^{-2}. However, cell cultures pretreated with 05 U/ml insulin and then irradiated lacked a shoulder at 10 J · m^{-2} and were biphasic in response at higher doses of UV. The toxic effect of increasing UV doses was measured more sensitively by relative cloning efficiency (RCE) than by the trypan blue dye exclusion technique; it took roughly a 10-fold higher dose to demonstrate a measured effect (Fig. 2) by dye exclusion than by inhibition of colony formation (Fig. 3). The slopes of each of the survival curves were similar (Figs. 2 and 3). The dose-

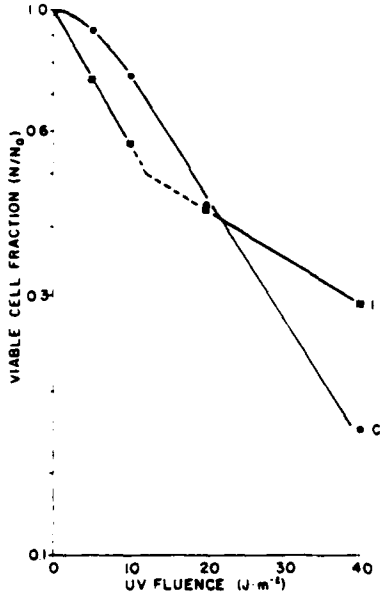

Fig. 2. Low PDL of NFS cells were seeded at 5000 cells · cm^{-2} into 60 mm diameter, 4-well plastic petri dishes and synchronized by amino acid deprivation. Following UV irradiation at a fluence rate of 1.2 J · m^{-2} · sec^{-1}, the plates were incubated at 37°C in a 4% CO_2 humidified air atmosphere. At this time cell suspensions were prepared by trypsinization and counted by trypan blue dye exclusion in a hemocytometer. The closed circles represent the irradiated cultures not exposed to insulin and the closed squares represent the irradiated cultures treated with 0.5 U/ml of insulin. Data points are the mean values for an *n* of 8.

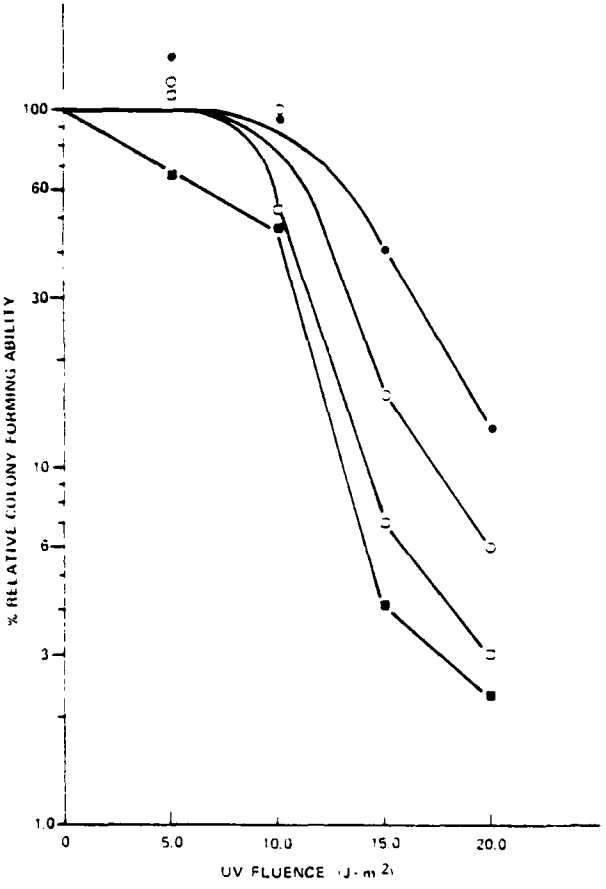

Fig. 3. Cells were seeded at 5000-cm^{-2} into a 60 mm diameter 4-well plastic petri dish containing Dulbecco's Modified Eagle's Medium supplemented with dialyzed FBS, minus arginine and glutamine (pH 7.2). These cells were released from the block (see text) and treated during the S phase with UV. Once the treated cells had completed S (8.2 h) they were seeded at a concentration of 200 cells/25 cm^2 well and cloned [3] for 11 days. The control (untreated) cultures exhibited 18—24% cloning efficiencies; results are reported as a percentage of control values. The circles (○——●) represent cells treated with UV. The squares (□——■) represent cells treated with UV and 0.5 U of insulin. The open circles and squares represent time points 12 h posttreatment at the conclusion of the S + G_2 + M part of the cell cycle, while the closed circles and squares represent time points coincident with the time points used for the trypan blue experiments (Fig. 1). Each point represents data from 10 different wells.

response profiles of cultures not treated with insulin were similar to those reported by Lehman et al. [11] or Maher et al. [12] for human skin fibroblasts. We noticed that when insulin-treated cells were irradiated at 5 J · m^{-2} (Fig. 3) in S phase there was an increased survival rate of up to 120% over the irradiated insulin-free cultures.

Synchronized cell populations that were not treated with either insulin or irradiated with UV exhibited an 18% RCE. Randomly proliferating cell populations treated with insulin-UV or UV alone, or synchronized cells treated with UV alone, exhibited a shoulder at 10 J · m^{-2} (Fig. 3). Insulin treatment appeared to increase sensitization of synchronized cell populations to UV treatment at 10 J · m^{-2} up to 20 J · m^{-2}. The insulin-UV-treated cultures at 20 J · m^{-2} exhibited a RCE of 3%, compared to 6% for cells not treated with insulin.

Growth characteristics of UV-treated cells: selective process

After 10–15 PDL in the 8% growth medium, the irradiated cultures (10 J · m^{-2}) appeared pleomorphic and contained more than 95% small polygonal cells. Many of these cells were multinucleated and displayed multiple processes as seen by Contrast Interference Nomarski microscopy. Cells passaged from these cultures proliferated to saturation densities of 4–6 × 10^6 cells 75 cm^2 flask in 8% growth medium, whereas untreated NFs cells only reached 1.2–1.5 × 10^6 cells 75 cm^2 flask. Treated cells (5 to 13.0 J · m^{-2}) exhibited a loss of contact inhibition, a tendency to pile up in culture and a pattern of irregular criss-cross growth (Table III). None of these altered characteristics were observed in control cultures. These cell populations were capable of subpassage to 120 PDL while the controls phased out after passage to 40 ± 5 PDL. Cell populations treated at a subtoxic dose of 1.2 J · m^{-2} exhibited no abnormal colony morphology.

To examine the cultures for growth at elevated temperatures, populations were subpassaged 1 : 4 and incubated at 37°C in a humidified 4% CO_2 atmosphere to allow cell attachment to the substratum. The temperature was then raised to 41°C. As early as 10 PDL after irradiation, cell cultures derived from UV-treated populations actively proliferated at 41°C for 72 h, at which time they were 60–70% confluent. Control cells detached from the surface in less than 24 h.

Growth in reduced serum

Many transformed mammalian cells have been reported to grow in reduced serum concentrations [13–15]. We examined our control and UV-treated human cell populations for this characteristic after 10 PDL by subpassing them into MEM supplemented with 1% FBS. Table I represents the growth characteristics of these cultures at reduced serum levels. Control NFS cells ceased proliferating after 5 PDL. After an initially slow growth rate, the UV-treated cultures appeared to adjust to the low serum concentration, as shown by the reduction in time needed to attain confluency. They continued to replicate at least through 17 PDL in 1% FBS then were transferred back to MEM + 10% FBS.

TABLE I

GROWTH OF CONTROL (NFS) AND UV TRANSFORMED HUMAN CELLS AT REDUCED SERUM CONCENTRATION

NFS cells PDL 14) and UV treated PDL 17 passages were transformed into MEM supplemented with 1% FBS and cellular proliferation was monitored. The NFS cultures ceased dividing after 10 PDL however, the cells from UV-treated culture appeared to adapt to the low serum concentration

Split ratio		Days to confluency		PDL	
NFS	UV	NFS	UV	NFS	UV
1:10	1:10	7	7	3.3	3.3
1:3	1:3	12	15	3.3	3.3
1:4	1:4	9	9	2	2
1:4	1:4	9	9	2	2
1:2	1:4	Dead	9	—	2
	1:4		7		2
	1:4		6		2
Total				10.6	10.6

Agglutination properties of UV treated cells

After at least 10 PDL in N1 growth medium, control and UV-treated cells were tested for agglutinability by exposure to varying concentrations of wheat germ lectin. UV-treated cells were agglutinated by 78 μg/ml of lectin, whereas control cultures of NFS cells required 2500 μg/ml, more than 30× as much [16], as the UV treated cells

Abnormal colony formation and growth in soft agar

Transformed cells require growth in culture before they will grow in soft agar. UV treated cell populations were serially passaged prior to seeding in soft agar. This was in contrast to serially passaged chemical carcinogen treated cells 16–20 PDL [1]. When UV-treated cells were serially passaged through 0.35% soft agar, the average colony frequency was 20 colonies/10^5 seeded cells. None of the untreated cells would grow in soft agar. Occasionally we observed small colonies of 2–6 cells in size, but they would not produce colonies 50 cells or larger and were not viable when isolated from soft agar after a 14-day incubation period in soft agar (Table II). Of 20 different cultures treated with $UV_{254\,nm}$ irradiation, we transformed 18 of them. Colony formation in soft agar was a measurement of transformation. The other 2 were refractory to the treatment. We therefore examined the cultures for variations in response to UV irradiated as a function of their ability to exhibit anchorage independent growth. We correlated this parameter with the frequency of abnormal colony formation and found that 5 J·m^{-2} induced the formation of a higher proportion of colonies (transformants) that grow in soft agar than 10 J·m^{-2}, while 10 J·m^{-2}

TABLE II

FREQUENCY OF COLONY GROWTH IN 0.33% AGAR OVERLAY OVER A 2.0% AGAR BASE

The data presented here is an average value for colony formation for 8 wells. Each well was seeded with 50 000 cells at PDL 20 into 0.33% agar over a 2.0% agar base. The colonies were counted after 21 days and the frequency of colony growth normalized to 100 000 cells per well.

Exp. No.	PDL	Growth in soft agar
1	20	20.0
2	20	15.0
3	20	25.1

induced the formation of a higher percentage of abnormal colonies (Table III). Cultures treated at a subtoxic dose of 1.2 $J \cdot m^{-2}$ exhibited no abnormal colony morphology and the treated cells would not grow in soft agar.

Tumor formation

The nodules which developed at the injection site in nude mice grew to be 0.6—1.05 cm in diameter and were well-encapsulated by mouse fibroblasts (Fig. 4). Removal of the tumors followed by karyological examination [1] confirmed that they were of human origin. The centers of the nodules were necrotic with dense infiltration of neutrophils and occasionally contained areas with extensive cholesterol cleft formation. Blood vessels

TABLE III

COMPARISON OF TOXIC EFFECT OF UV_{254nm} IRRADIATION WITH FREQUENCY OF ABNORMAL COLONY FORMATION AND ANCHORAGE INDEPENDENT GROWTH

UV ($J \cdot m^{-2}$)[a]	% Inhibition of colony formation	Abnormal colony[b] morphology 10^3 cells	Grow[c] in spec. ac.
1.2	0.0	0.0	0.0
5.0	0.25	2.1	$10^{-1.4}$
10.0	0.50	3.5	$10^{-0.7}$
13.0	0.60	0.5	10^{-1}
13.5	0.65	0.5	0.0
16.0	0.95	0.0	0.0

[a] The above exposures were administered at 1.2 $J \cdot m^{-2} \cdot sec^{-1}$.

[b] After exposure to UV at the conclusion of S the cells were seeded at 10^3 cells/25 cm^2 well and cloned [9]. At the end of 9 days they were fixed, stained and enumerated.

[c] After 20 PDL, 50 000 cells were seeded in 2 ml of 0.33% agar. The cultures were refed once a week for 17 days. At that time the colonies were counted and the frequency of colony growth in soft agar reported as the number of colonies containing 50 or more cells. 10^3 cells seeded.

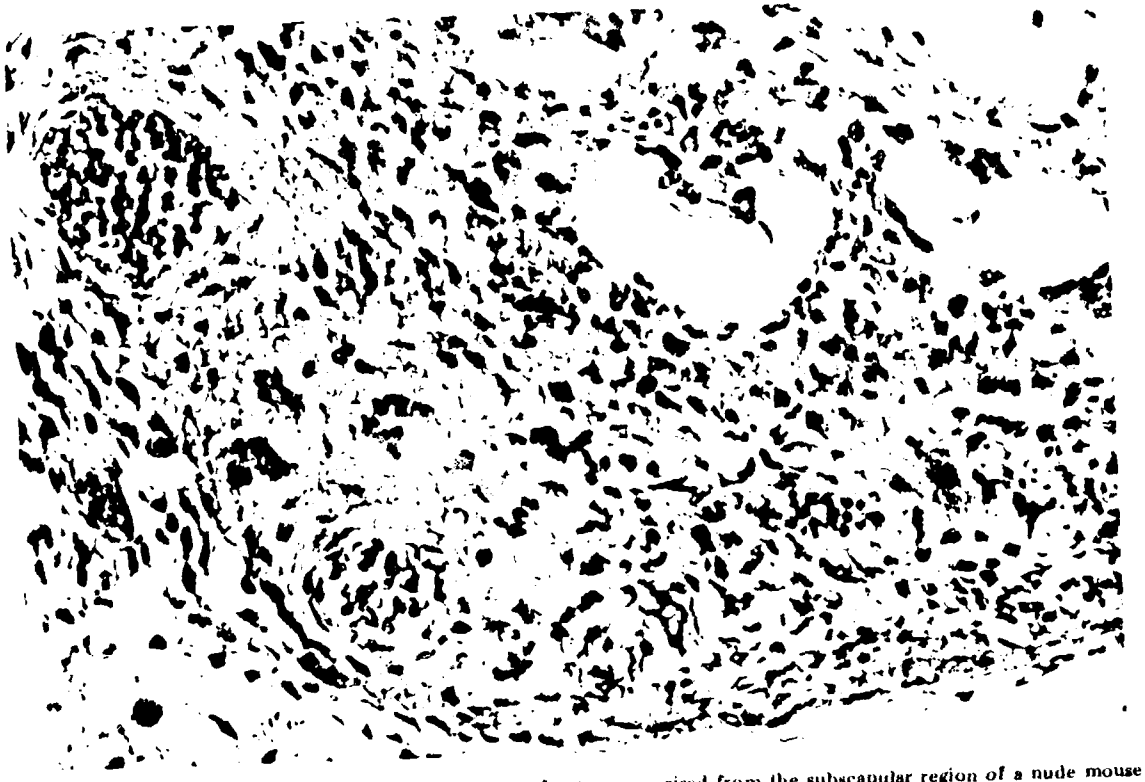

Fig. 4. Photomicrograph (120×) of a serial section of a tumor excised from the subscapular region of a nude mouse. The tumor was excised 4 weeks after injection of 5×10^6 cells. The tumor was fixed in buffered formalin and stained with hematoxylin and eosin.

were present within the nodules. The nodules were compatible with a benign proliferation of exogenous cells and could be described as myxofibroma-like.

Of 6 mice innoculated with the same inoculum, 4 developed tumors. After 4—6 weeks of nodule growth, the animals were sacrificed by cervical dislocation and the nodules removed for histopathology. These experiments were repeated 4× with frequencies of tumor formation of 4/6, 3/6, 5/6 and 3/6. They were fixed in 10% formalin, embedded, sectioned and stained with hematoxylin and eosin. To date, similar results have been obtained in 8 successive attempts while 200 irradiated untreated non-inoculated animals have not formed tumors. Fifty mice received a cell pack of 5×10^6 cells from 5 different lines and from 5 different tissues. In all the mice the bleb regressed within 72 h and the mice died 1—1.5 years later of natural causes. At autopsy there was no evidence of tumor at the site of injection of the cell pack.

DISCUSSION

Several parameters of putative changes in human foreskin cell populations during passage from the early stages of the transformation process to the late stages (growth in nude mice) were evaluated. Characteristics associated with UV-irradiated cell populations as early as PDL 5 and after discontinuation of the carcinogen treatment were altered colony morphology [1], altered saturation density [1] growth at 41°C and growth in 1% FBS-supplemented growth medium. Altered colony morphology was observed within 3 PDL after discontinued treatment. Immediately after the completion of S (8.2 h in length) the cells were split 1 : 10 and part of the treated cell population was cloned at 1000 cells · cm^{-2}. We observed colonies in the flasks or wells that exhibited a loss of contact inhibition and a criss-cross, disoriented piling up of the cells. These populations, when isolated from the rest of the colonies in the culture, gave rise to cell populations that exhibited abnormal morphology and an absence of the long parallel wholing growth patterns in normal untreated cultures. It was from these abnormal colonies that the transformed cell types (exhibit anchorage independent growth) arose. We observed 2—4 per 25-cm^{-2} well. In 8 treated wells we found 2 abnormal colonies that would grow in soft agar. Individual abnormal colonies also have to be serially passaged for 16—20 PDL before they would grow in soft agar. In each situation the frequency of growth in soft agar was similar; $10^{-3.5}$. In human cell transformation studies, abnormal colony morphology, while important as a tool to aid in determining if a transformation event happened, was not a reliable marker to quantitate the transformation events. In the refractory cell populations that would not exhibit anchorage independent growth, no abnormal colonies were observed during the early stages of the expression stage. These same phenomena have been observed for chemical carcinogen treated cell populations.

After 10 PDL, irradiated cell populations exhibited an alteration in lectin

agglutination profiles and grew at a temperature of 41°C. In fact, we found that 2—3 serial subpassages could be manipulated at 41°C. Normal cells will not survive 24 h at that temperature. However, at this point the cells still would not grow in soft agar, nor were they able to produce tumors in nude mice. Serial passage to 20 PDL was required to produce populations that would grow in soft agar.

After passage through soft agar and tumor formation in the nude mouse, UV-treated cell populations were able to grow to a finite PDL of 120—140. Cells with a normal phenotype phased out at PDL 40 ± 5. It is interesting to note that UV light has been shown to induce carcinogenesis in the skin of man although it has weak penetrating ability. UV light can be differentiated from X-ray by the specific nature of the induced damage and DNA repair processes. Owing to its particular features UV light, unlike many chemical carcinogens, does not require antecedent metabolic activation or specific binding before an interaction with cellular DNA.

The slopes of the toxicity profiles (survival curves) are similar to those seen for 10 T1/2 cells [17] on a per cell basis (Fig. 2). When we evaluated the survival response patterns of cells at risk 12 and/or 40 h after initiating treatment (4—8 h prior to the reinitiation of scheduled DNA synthesis), we found that by growing the cells at a low cell density the toxicity slopes (Fig. 3) were very similar to the slopes measured on a per cell basis (Fig. 2). We did note the presence of abnormal colonies and it was from these colonies that the neoplastically transformed cell populations arose. If we plotted the numbers of these abnormal colonies as a function of the dose in $J \cdot m^{-2}$ recorded as a frequency relative to the number of normal colony phenotypes, the optimum numbers of transformants occurred at a survival dose of 50 (ED_{50}) (Table III). At an ED_{65} the frequency of abnormal colonies was zero. The frequency of abnormal colonies we observed at ED_{25} was $10^{-3.6}$; at an ED_{50} it was $10^{-3.2}$; at an ED_{60} it was 10^{-5} (Table III). The same observations have been made for cell populations treated with chemical carcinogens of the type whose action does not require activation [1]. As in animal cell systems, treated human cells serially passage 24 h after exposure of the cells to UV, to give them time to replicate, enhances the formation of abnormal foci. .

Therefore, in order to produce cell populations in the initial stages of a transformed phenotype the transformed cells must be allowed to replicate. When the treated cells were permitted to remain in a confluent density or non-proliferating phase of growth after treatment, no transformed cell populations were obtained. Contrary to the observation by Little [18] that survival is enhanced when mouse cells are left in growth-inhibited conditions after X-irradiation, human cells at that point are lost from the transformed phenotypic populations. However, the rate of repair processes in rodent cells is much slower than in human cells, which may explain why rodent cells must be allowed a longer repair time before they replicate and establish a transformed phenotype.

Since we can optimize the transformation events by irradiating NFS cells

in S, while treatment in G_1, or G_2 or M minimizes the events (Milo and DiPaolo, unpublished data), we believe that the process of UV-induced carcinogenesis in human fibroblast cells is complex, error prone and subject to critical timing of exposure to UV at an appropriate dose of the physical carcinogen. Following a selection process, the expression stage can be controlled by allowing at least 20 additional rounds of proliferation to occur before the cells will passage through soft agar and form tumors in the mouse.

In conclusion, we agree that the rate of recovery of cloning ability of human fibroblasts after UV treatment shows a correspondence with the ability of the cells to recover from the potential lethal effects of the irradiation [6]. However, we have found that even though we can demonstrate UV-induced transformation at toxic doses of ED_{50} or less, we cannot demonstrate transformation at higher toxic doses or lower non-toxic doses. Therefore, we feel that cell populations contain subsets of cells that are susceptible to carcinogen(s) but are lost from the total cell populations by modulation of the normal phenotype upon prolonged exposure to the tissue culture environment.

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REFERENCES

1 G.E. Milo and J.A. DiPaolo, Neoplastic transformation of human diploid cells in vitro after chemical carcinogen treatment, Nature, 275 (1978) 130.
2 G. Milo, R. Olsen, S. Weisbrode and J. McCloskey, Feline sarcoma virus induced in vitro progression from premalignant to neoplastic transformation of human diploid cells, In Vitro, 16 (1980) 813.
3 B. Sutherland, J. Arnino, N. Delihas, A. Shih and R. Oliver, Ultraviolet-light-induced transformation of human cells to anchorage-independent growth, Cancer Res., 40 (1980) 1934–1939.
4 H.F. Stich, R.H.C. San, J.A. Miller and E.C. Miller, Various levels of DNA repair synthesis in *Xeroderma pigmentosum* cells exposed to the carcinogens N-hydroxy and N-acetoxy-2-acetylaminofluorene, Nature (New Biol.), 238 (1972) 9.
5 V.M. Maher, N. Birch, J.R. Otto and J. McCormick, Cytotoxicity of carcinogenic aromatic amides in normal and *Xeroderma pigmentosum* fibroblasts with different DNA repair capabilities, J. Natl. Cancer Inst., 54 (1975) 1287.
6 R.H. Heflich, R.M. Hazard, L. Lommel, J.D. Scribner, V.M. Maher and J. McCormick, A comparison of the DNA binding, cytotoxicity and repair synthesis induced in human fibroblasts by reactive derivatives of aromatic amide carcinogens, Chem.-Biol. Interact., 29 (1980) 43.
7 J. McCloskey and G. Milo, In vitro transformation of normal diploid human cells by UV and X-rays. Abstracts. Fifth Annual Meeting of American Society for Photobiology, San Juan, Puerto Rico, 1977, p. 110.
8 D.A. Reigner, T. McMichael, J.C. Berno and G.E. Milo, Processing of human tissue to establish primary cultures in vitro, Tissue Culture Ass. Manual, 2 (1976) 273.
9 G.E. Milo, W. Malarkey, J. Powell, J. Blakeslee and D. Yohn, The effects of steroid hormones in fetal calf serum on plating and cloning of human cells in vitro, In Vitro, 12 (1976) 23.

10 J. Oldham, L.E. Allred, G.E. Milo, O, Kindig and C.C. Capen, The toxicological evaluation of the mycotoxins T-2 and T-2 Tetraol using normal human fibroblasts in vitro, Toxicol. Appl. Pharmacol., 52 (1980) 159.
11 A.R. Lehman, S. Kirk-Bell, C.F. Arlett, S.A. Harcourt, E.A. deWeerd-Kastelein, W. Keijzer and P. Hall-Smith, Repair of ultraviolet light damage in a variety of human fibroblast cell strains, Cancer Res., 37 (1977) 904.
12 V. Maher, J.J. McCormick, P.L. Gover and P. Sims, Effect of DNA repair on the cytotoxicity and mutagenicity of hydrocarbon derivatives in normal and *Xeroderma pigmentosum* human fibroblasts, Mutat. Res., 43 (1977) 17.
13 Y. Oshiro and J.A. DiPaolo, Loss of density-dependent regulation of multiplication of BALB/3T3 cells chemically transformed in vitro, J. Cell. Physiol., 81 (1973) 133.
14 H.M. Temin, Control of cell multiplication in uninfected chicken cells and chicken cells converted by avian sarcoma viruses, J. Cell. Physiol., 74 (1969) 9.
15 C. Borek, Neoplastic transformation in vitro of a clone of adult liver epithelial cells into differentiated hepatoma-like cells under conditions of nutritional stress, Proc. Natl. Acad. Sci. U.S.A., 69 (1972) 956.
16 G.A. van Nest and W.J. Grimes, Concanavalin A-induced agglutination and tumorigenicity in virally and spontaneously transformed cells derived from BALB/c mice, Cancer Res., 34 (1974) 1408.
17 J. Little, Radiation carcinogenesis in vitro: implications for mechanisms, in: H. Hiatt, J. Watson, and J. Winston (Eds.), Human Cancer, Cold Spring Harbor Lab. Publ. Co. Book B, (1972) 923.
18 J. Little, The role of cell division in the malignant transformation of mouse cells with 3MCA, Cancer Res., 35 (1975) 1637.

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PRESENSITIZATION OF HUMAN CELLS WITH EXTRINSIC SIGNALS TO INDUCED CHEMICAL CARCINOGENESIS

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Foreskin-derived low-passage human cell populations were reproducibly transformed with chemical carcinogens when the cells were blocked in G_1, released from the block, and treated with either the carcinogen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) or with Aflatoxin B1 in the S period of the cell cycle. Arginine- and glutamine-deficient medium was required to effectively block the cells in the G_1 period. Estradiol, insulin, anthralin or phorbol myristate acetate sensitized the cell population to carcinogen treatment when added 10 h before the carcinogen in early S period. Presensitized cells kept blocked in G_1 period for 48 h or longer, released and treated in S period with MNNG or Aflatoxin B1 were not transformed; nor did transformation occur in presensitized cell populations treated in G_1 (4.5 h), M (1.5 h) or G_2 (8.2 h). Cells derived from carcinogen-treated presensitized cells grew as colonies in soft agar at 16-20 PDL. When cells derived from colonies isolated from the soft agar were injected subcutaneously into nude mice, tumors developed.

Serially subpassaged human cells grow *in vitro* as randomly proliferating monolayer cultures with subpopulations capable of different rates of scheduled (Cristofalo and Sharf, 1973) and/or unscheduled DNA repair synthesis (Hart and Setlow, 1976). With continued subpassaging the non-proliferating populations constitute a progressively larger proportion of the total cell population. These changes in cell cycle kinetics with serial subpassaging could be due to a decrease in proliferating subpopulations (Cristofalo and Sharf, 1973; Merz and Ross, 1973; Turk and Milo, 1974; Milo and Hart, 1976) or to a lengthening of the G_1 or G_0 cell cycle phase (Grove and Cristofalo, 1977). In the terminal passages of Phase III, unscheduled DNA repair synthesis also decreases in subcultured human diploid populations (Milo and Hart, 1976).

The expression of the transformed phenotype after a carcinogenic insult requires preferential cell multiplication. The failure of cell proliferation and fixation of the initial transformation event result in the chemically treated cells becoming part of the cell population belonging to a permanent resting phase of the cell cycle. In this way, prevention or suppression of cell transformation would occur.

We have previously demonstrated that chemical carcinogen-induced transformation of human cells occurs in low-passage populations that are first blocked in G_1, released from the block, then treated in S (Milo and DiPaolo, 1978). The current study shows that several chemicals may sensitize cells and alter the subsequent transformation response of human cells to chemical carcinogens.

MATERIAL AND METHODS

Cell cultures

Neonatal human foreskin cell suspensions obtained by collagenase dispersion (Riegner *et al.*, 1976), were seeded into 25-75 cm^2 flasks and produced confluent monolayers within 48 h. These cell cultures have a finite replicative capacity of 35 ± 7 population doubling (PDL). Cultures containing rapidly proliferating cells (Cristofalo and Sharf, 1973) were arbitrarily assigned to level 2 after the first subpassage. All populations were routinely passaged on Eagle's minimum essential medium (MEM) supplemented with non-essential amino-acids, sodium pyruvate, gentocin, glutamine, 25 mM Hepes at pH 7.2 (Milo and DiPaolo, 1978) and 10% fetal bovine serum (FBS). All FBS used in the above complete medium (CM) for these experiments was first analyzed for content of hydrocortisone (HC), 17-β-estradiol (E_2), progesterone (P), cortisone (C) (Milo *et al.*, 1976), and unsaturated fatty acids (Huttner *et al.*, 1978). FBS selected for the carcinogenesis-synchronization experiments optimally exhibited growth potential of 40-50% colony-forming efficiency in cultures seeded at 250 cells/25 cm^2 flask.

G_1 period

To block the human cells in the G_1 period, several variations of Dulbecco's modified minimum essential medium (DM) deficient in specific amino-acids were used: lacking either leucine-glutamine, isoleucine-glutamine, leucine-arginine, isoleucine-arginine, or glutamine-arginine combinations, or one of the individual amino acids. The technique used to block the cells in the G_1 period was modified from the Tobey and Ley procedure (1971). Cell suspensions of 5,000 cells/cm^2 were seeded onto four microscope slides and placed in 176-cm^2 Petri dishes containing 50 ml of each amino-acid-deficient DM

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Abbreviations: Population doubling, PDL; fetal bovine serum, FBS; complete medium, CM; hydrocortisone, HC; 17-β-estradiol, E_2; progesterone, P; cortisone, C; Dulbecco's modified minimum essential medium, DM; Minimum essential medium, MEM; specific activity, S.A.; 9-octadecenoic acid (oleic acid), C 18:1; arachidonic acid (eicosatetraenoic acid-5,8,11,14), C 20:4; dibutyryl cyclic GMP, d-cGMP; insulin, IN; phorbolmyristate acetate, PMA; anthralin, Anth; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, MNNG; human nasopharyngeal carcinoma cell line, NPC; SV-40 transformed WI-26, VA-4; Eagle's basal medium, EBME; Low calcium-Eagle's basal medium; LoCal-EBME.

medium supplemented with dialyzed 10% FBS (d-FBS). Cells were incubated in a 4% CO_2-enriched air atmosphere at 37°C. The cultures were released from the G_1 period block by exchanging the amino-acid-deficient medium to growth medium (CM) consisting of Eagle's MEM 10% FBS, with the addition of [^{3}H]thymidine (1 μCi/5 ml) and, where appropriate to the experiment, an extrinsic factor such as IN. The radiolabelling period for continuously labelled cells was 96-144 h (S.A. 6.0 Ci/mmole of [^{3}H]thymidine) or for pulse-labelled cells, 30 min (S.A. 60 Ci/mmole of [^{3}H]thymidine). The CM, including radiolabelled thymidine, was replaced every 24 h in the continuously labelled culture. After the block was removed, chemicals known to alter cellular DNA synthesis were suspended in CM and added to the cell population at either 24, 48, 72, 96 or 120 h.

Chemicals

The extrinsic factors studied for their effect on the S phase of the cell cycle were oleic acid (9-octadecenoic acid (C18:1); arachidonic acid (eicosatetraenoic acid - 5, 8, 11, 14 (C20:4) (Nu-Chek, Elyrian, Minn.; Huttner *et al.*, 1978) at 5 μg/ml; dibutyryl cyclic GMP (d-cGMP) at 2.07 μg/ml; insulin (IN) at 0.5 U/ml; 17-β-estradiol (E_2) at 1 μg/ml; hydrocortisone (HC) (Schwartz/Mann, Orangeburg, N.Y.) at 1 μg/ml; phorbol-myristate acetate (PMA) (Consolidated Midland Corp., Brewster, N.Y.) at 1 μg/ml; and anthralin (Anth) (Pfaltz and Bauer, Inc., Stamford, Conn.). Chemicals were dissolved in acetone (Spectrar Grade, Mallincrodt, Inc. Paris, Kentucky) under red light in an argon atmosphere, and maintained in stock solutions of 1 mg/ml. The carcinogens, aflatoxin B_1 and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), were dissolved in acetone and stored at −19°C until immediately prior to use. The final culture concentration of acetone was 0.02%. Cell cultures that had been pulsed with [^{3}H]thymidine (60 Ci/mmole) for 30 min, or continuously (6.0 Ci/mmole) for 120 h, were acid-washed with 0.1 N HCl to remove free [^{3}H]thymidine. The culture slides were fixed in Carnoy's solution or methanol: acetic acid (3:1, v/v), dipped in Kodak NTB-2 emulsion, exposed for 3-4 days at 12°C, developed in Kodak D-19 developer, fixed in Kodak fixer 197-1746, and stained with filtered Giemsa.

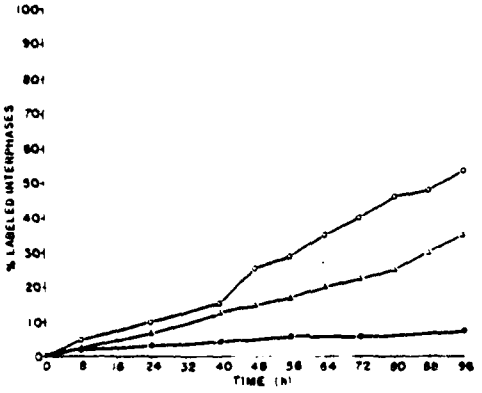

FIGURE 1 — Human foreskin cell populations were seeded at a cell density of 5,000 cells/cm² onto a 15-mm diameter microscope slide or a coverslip. These seeded slides were then placed into 25-cm² dishes containing 5 ml of DM supplemented with 10% d-FBS minus arginine-glutamine (●—●), minus leucine-glutamine, (△—△), or minus isoleucine-glutamine (○—○), and monitored for the presence of radiolabelled interphases for 96 h after seeding. The deficient medium was replaced every 24 h. [^{3}H]thymidine (1 Ci/5ml; S.A. 6.0 Ci/mmole) was added at the time when the cells were seeded into the DM. The radiolabel was replaced every time the DM or CM was replaced. Four samples were taken at 1 h over the 96-h period, fixed and prepared for autoradiography (see text).

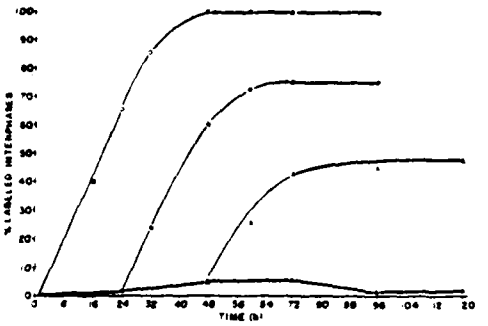

FIGURE 2 — Human foreskin cell populations at saturation density were seeded at a cell density of 5,000 cells/cm². These populations were not permitted to remain in saturation density for more than 16 h prior to seeding. The cells were suspended by trypsinization (Riegner *et al.*, 1976) and the cells recovered by centrifugation at 650 *g*. The pellet was resuspended into experimental medium A and seeded onto 15-mm diameter microscope sides or coverslips for 6 h to attach to the substratum. These cultures were kept in this deficient medium for 24 h (●—●) (see legend to Figure 1) or 48 h (△—△). The block was removed by the addition of 10% FBS-supplemented CM containing 2 mM arginine and 1 mM glutamine. One μCi of [^{3}H]thymidine (6.0 Ci/mmole)/5 ml was added to each amino-acid-deficient experimental medium and each complete growth medium. In each case, both medium and radiolabelled thymidine were replaced at every 24-h period from seeding to completion of the experiment. Cells kept continuously in CM and serially subpassaged into CM were sampled every 2 h from 0 h up to 96 h (○—○) post seeding. Proliferating cell populations were seeded into experimental medium A for 24 h (●—●); four samples were removed every 2 h from 0 h (at 24 h) up to 96 h. The block was removed at 24 h. Additional populations were left in experimental medium up to 48 h (△—△) then the block was removed. Another population was left in the amino-acid-deficient medium from 0 h up to 120 h (▲—▲). The medium plus [^{3}H]thymidine was replaced every 24 h.

Preparation of metaphase chromosomes

Rapidly proliferating cell cultures at PDL 1-5 were prelabelled with 1 μCi/ml [^{3}H]thymidine (6.0 Ci/mmole) for 24 h. Grains were found on 100% of the interphase nuclei. Companion cultures radiolabelled in the same manner were fed every 24 h with amino-acid-deficient DM (pH 7.2) supplemented with 10% d-FBS. Sixteen to 24 h later the deficient medium

was replaced with CM; 0.1 μg/ml colcemide was added 3 h prior to fixation. Samples were removed every 30 min beginning 4 h into the S phase, fixed in Carnoy's solution and either stained with aceto-orcein before being dipped in Kodak NTB-2 emulsion or post-stained with Giemsa following development (Baserga, 1967) 4 days later in Kodak D-19 developer and fixation in Kodak fixer 197-1746.

Growth in soft agar

Two transformed cell lines were used as positive controls for evaluating the growth potential of chemical carcinogen-treated cells in soft agar. These were a human nasopharyngeal carcinoma cell line (NPC; gift from Litton Bionetics, Kensington, Md.) and an SV-40-transformed lung cell line (VA-4; from the A.T.C.C., Rockville, Md.). Both grew in soft agar (0.35% containing LoCal-EBME-supplemented with 20% FBS) over a 1% or 2% agar base containing RPMI 1629 growth medium supplemented with 20% FBS (Milo and DiPaolo, 1978). The frequency of bolus formation with NPC and VA-4 after 9-11 days was 70-80% and 80-85%, respectively. The chemically treated cells were seeded at 50,000 cells/25 cm^2 well (Milo and DiPaolo, 1978) and examined 11 days later.

Growth in nude mice

Nude mice, purchased from Sprague-Dawley (Madison, Wisc.) were delivered at 6 weeks of age. Mice between 10 and 12 weeks of age, previously subjected to 450 R whole-body irradiation, were inoculated subcutaneously with 10^6-$10^{7.5}$ NPC-cells per inocolum into the subscapular region. After 24 h the initial bleb regressed and 4-6 weeks later a tumor 0.6-1.2 cm in size was excised. The optimum inoculum size was 5×10^6 cells. VA-4 cell inocula regressed in 24 h and no visible tumor was seen in the 20 inoculated mice after a 6-month period. The tumor incidence in NPC-inoculated mice was 5/10. The chemical carcinogen-treated cell populations were inoculated at a cell inoculum size of 5×10^6 cells in 0.5 ml volume in the manner previously described and submitted for pathology (Milo and DiPaolo, 1978). After 4-6 weeks the tumors were removed and submitted to pathology.

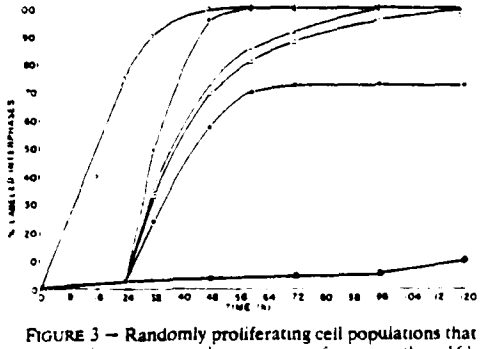

FIGURE 3 — Randomly proliferating cell populations that were not in saturation density arrest for more than 16 h were seeded into CM (○—○) or medium A (■—■) and allowed to attach for 6 h (>95% attachment was determined by fixation, staining and counting the number of cells that attached to the substratum versus the number of cells seeded). All populations seeded into medium A were transferred to CM plus 2 mM arginine and 1 mM glutamine and either HC, C20:4, E_2 (▲—▲); or IN (△—△); or PMA (●—●); or Anth (●—●); or d-cGMP (□—□) added to each separate culture at 24 h. [^{3}H]thymidine was added to the cultures as described in the legend to Figure 2. Cultures were re-fed every 24 h over the 120-h period.

RESULTS

Cell block

Cells from amino-acid-deficient DM preparations supplemented with 10% d-FBS contained varying numbers of radiolabelled nuclei (Fig. 1). At 96 h cells maintained in DM minus glutamine and arginine (Medium A), minus leucine and glutamine (Medium B), or minus isoleucine and glutamine (Medium C) contained 5, 37 and 57% radiolabelled interphase cells, respectively.

In DM-deficient media lacking only one amino acid (*i.e.*, arginine, glutamine, leucine or isoleucine), the cell populations contained 50-70% radiolabelled interphases over a 96-h period. A 65% increase in cell numbers 10 h after the S period corresponded to the number of radiolabelled interphase nuclei observed.

Medium A was selected for blocking the cells in G_1, and samples were checked at 2-h intervals. Cell populations seeded for 24 h in medium A and transferred to CM for 72 h contained 67-72% radiolabelled nuclei. Cells kept in medium A for 48 h prior to transfer to CM contained 35-42% radiolabelled nuclei at the end of an additional 72 h period. The area in Figure 2 between the curves for randomly proliferating cell populations (open circles) and the cells held in medium A for 24 h (filled circles) represents cells that respond to IN, Anth, E_2, or PMA treatment (see below). The area described by the cell population that was held in medium A for 48 h (Fig. 2, triangles) represents a population that will not totally respond to added signals such as IN, Anth, or E_2.

Effect of exogenous factors on cell growth

Cell populations were transferred from medium A to CM after 24 h and one of the following chemicals was added: IN, HC, C20:4, E_2, d-cGMP, PMA, or Anth (Fig. 3), along with [^{3}H]thymidine. In these experiments, untreated control cultures transferred to CM after 24 h in medium A contained radiolabel in only 62-67% of cell nuclei up to 120 h after transfer. Either IN, C20:4, E_2, d-cGMP or HC added to CM amplified the appearance of radiolabelled nuclei over the 120-h sampling period. Anth or PMA did not alter the profile of [^{3}H]thymidine incorporation into nuclei. IN-treated cells recovered from the block more rapidly than any of the other cultures. Whenever treated cultures were removed from the experimental medium and passaged, the normal proliferative kinetics resumed by passage 2 or 4. The PDL time after a 1:2 split was approximately 2-3 days at PDL 2. In no case was the lifespan of cultures appreciably altered from 37±7 PDL.

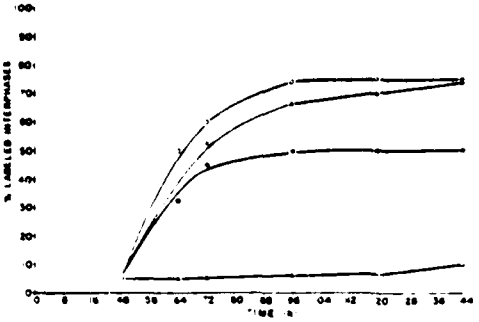

FIGURE 4 — Cell populations used in these experiments were prepared and seeded in the same manner as described in the legend to Figure 3. However, these cultures were released from block after 48 h. [^{3}H]thymidine was added to medium A and/or CM as described in the legend to Figure 2. Control cultures (●—●) were released after 48 h and IN (○—○) or HC (▲—▲) was added. Other compounds listed in the legend to Figure 3 induced responses intermediate between those of IN- (○--○) or HC- (▲--▲) treated cultures. These compounds were added to CM 48 h after replacement of medium A. The untreated control cultures were left in medium A (△—△). [^{3}H]thymidine was added to the cultures at seeding and every 24 h upon replacement of medium A or CM.

To further study the effect of these compounds on the proliferative capacity of the cells, populations were maintained in medium A for 48 h (Fig. 4) before treatment. Untreated control cells in these experiments contained 43% radiolabelled nuclei 48 h after transfer from medium A. IN-treated cell populations contained 72% radiolabelled nuclei. C20:4- or HC-treated cultures sampled for up to 120 h incorporated [^{3}H]thymidine into no more than the 72% of nuclei labelled at 48 h. Again, in Anth-or PMA-treated cultures the labelling profile did not differ from that of the untreated cultures.

Since it was difficult to predict the exact length of the lag interval before the cells in G_1 would begin to enter the S period following transfer from medium A to CM, the cells were blocked in G_1 in medium A for 24 h and then released. At point B (Fig. 5) either IN or one of the other compounds (see Figs. 1-3) was added to the CM. At 30-min intervals for the next 25 h, sample populations on 1 mm were removed and placed in CM containing 1.0 μCi of [^{3}H]thymidine (60 Ci/mmole) in 5 ml for 30 min. Untreated control cultures achieved a level of only 73% radiolabelled nuclei, whereas cells treated with IN reached a 90-95% level.

When E_2, Anth or d-cGMP (Table I) were added to pulse-labelled cultures, the S peak occurred 1 h later. Compared to untreated controls, the length of S (*i.e.*, the appearance and disappearance of labelled interphase nuclei) did not vary in any of the treated populations, the length of S in all populations was 8.2 h. The length of M, 1.5 h, was determined by measuring the interval for the appearance and disappearance of radiolabelled metaphase plates. The interval between S and M (G_2) was calculated to be 4.5 h. The length of the cell cycle, 22.4 h, was determined by counting cells over a 24-h period using trypan-blue dye exclusion. Subtraction of the experimentally determined S and M times and the calculated time for G_2 from 22.4 yielded an estimated G_1 of 8.2 h. If either IN or Anth was added at interval B (Fig. 5), the period most dramatically affected was G_1 • G_1 was shortened from 8.2 to 6.5 h between waves of cells passing through S, G_2 and M for three cycles. After the first cycle and for each successive cycle 20-22% of the cells departed from the synchrony pattern. After the second cycle, the number of rapidly pulse-labelled nuclei decayed to that of randomly proliferating companion cultures. The absolute values for the total number of cells in S varied with tissue and according to treatment. When experimental sister cultures were treated with PMA, IN, Anth or E_2 at point B (Fig. 5), the number of cells in S varied from 67-95% (Table I).

TABLE I

PERCENT [^{3}H]THYMIDINE LABELLED NUCLEI DURING THE PEAK TIME INTERVAL OF SCHEDULED DNA SYNTHESIS OF CELL POPULATION TREATMENT WITH DIFFERENT EXTRINSIC FACTORS*

Time (h)	Treatment							
	C20:4	IN	HC	E_2	Anth	PMA	d-cGMP	Untreated
34	35	42	45	21	22	17	12	11
35	74	—	74	33	33	23	47	49
36	90	92	89	51	41	35	67	72
37	84	81	81	91	55	33	72	67
38	63	72	72	67	38	15	63	54
39	57	60	57	37	22	7	55	34

*Concentrations used were below the level that inhibited cellular proliferation as evaluated by relative plating efficiency of the cell population (Milo *et al.*, 1976).

†The cells at 5,000 cells/cm^2 were blocked in G_1 in DM minus arginine minus glutamine medium for 24 hrs., transferred to CM + 10% FBS containing either arachidonic acid, (C20:4), insulin, (IN), hydrocortisone, (HC), estradiol, (E_2), anthralin, (Anth), phorbol myristate acetate, (PMA), di-butyryl cyclic GMP, (d-cGMP) or untreated. Companion wells contained 5 ml of CM with one μCi/5 ml of [^{3}H]thymidine (60 Ci/mmole). At 30 minute intervals a sample was removed from DM or CM medium and incubated in CM + [^{3}H]thymidine for 30 minutes, removed, fixed, stained and developed. This procedure was carried out at hr. 34–39 post-seeding. The percent labeled nuclei is expressed as the number of radiolabeled interphases per total number of nuclei per 100 nuclei.

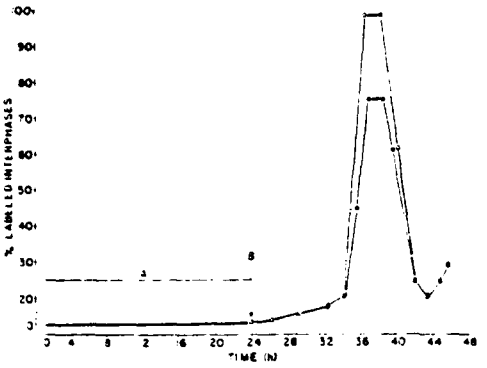

FIGURE 5 — Cell populations were seeded at a density of 5,000 cells/cm² with DM minus arginine-glutamine, (A) supplemented with 10% d-FBS. The cells were fixed 24 h later, stained with hematoxylin, and enumerated (continuously 95% absolute plating efficiency occurs). The DM was removed at this time and the cultures released with CM (see text) containing 0.5 U of insulin per ml. Companion wells containing 5 ml of CM with [³H]thymidine were incubated under the same culture conditions as for the experimental DM cultures. One μCi of [³H]thymidine (60 Ci/mmole) was added. At 30-min intervals samples were removed from DM or CM medium, incubated for 30 min in the radiolabelled CM medium, fixed, stained and developed under NTB-2 emulsion for 4 days. The labelled interphases were enumerated. Companion slides and labelling conditions were used in controls except that 0.1 μg/ml of colcemide was added to the CM medium. Four hours into S, samples were taken and radiolabelled for 30 min in CM, fixed, and developed under NTB-2 emulsion (see text). Radiolabelled metaphases were enumerated and the percentages compared to the controls were calculated (not reported here).

Cell transformation studies

Ten hours after administration of IN or a compound listed in Table I at interval B (as the cells were entering S; Fig. 5), aflatoxin B_1 or MNNG was added to the cultures. The carcinogens were removed 12 h later and the cultures serially passaged into CM containing 8× non-essential amino acids and 2× vitamins (Milo and DiPaolo, 1978), for 15 PDL. The populations were then serially passaged into soft agar (0.35%, containing LoCal-EBME supplemented with 20% FBS) over a 2% agar base containing RPMI 1629 growth medium supplemented with 20% FBS (Milo and DiPaolo, 1978). After 11 days colonies were scored, removed, and serially subpassaged in CM. Growth in soft agar for treated cultures ranged from no growth to $1:10^{2.5}$ for aflatoxin B_1-anthralin treated cultures (Table II). Cell populations treated with non-carcinogenic compounds and passaged through soft agar sometimes formed short chains of two to six cells rather than a colony. None of these cells formed colonies in agar when passaged a second time. Addition of IN, E_2, PMA or Anth at interval B enhanced colony formation in soft agar as compared with aflatoxin treatment alone. Serially passaging the cell a second time in the agar increased the frequency of colony formation to 40-90%. Similar results reoccurred with MNNG-treated cell populations regardless of whether IN or Anth were used (data not shown here). PMA-aflatoxin B_1-treated populations, while exhibiting a low frequency of colony formation upon passage through the soft agar for the first time, did form colonies of 50-300 cells per colony, followed by an increase in frequency in colony formation to 70% during the second agar passage.

Addition of oleic acid (C18:1), C20:4, or d-cGMP did not enhance colony formation. E_2 and PMA were intermediate in their effect on colony formation by carcinogen-treated cells, while IN or Anth were quite effective in augmenting colony formation (Table II). After carcinogen-treated cells isolated from agar were serially passaged, 5×10^6 cells were inoculated subcutaneously into the subscapular region of previously irradiated (450R whole-body) nude mice (Milo and DiPaolo, 1978). Subcutaneous tumors appeared at the injection site within 10-18 days. The tumors were scored 4 weeks later when 0.8-1.5 cm in size. Cell populations treated with aflatoxin B_1-IN or Anth, or with MNNG-IN or Anth, produced the highest numbers of tumors (Table III). In three experiments (data shown for one experiment in Table III), the order of successes was the same. Aflatoxin B_1-IN or MNNG-IN induction of tumors was the highest, followed by Aflatoxin B_1-Anth, or -PMA. Tumors excised and examined histopathologically and karyologically were all confirmed as undifferentiated mesenchymal tumors of human origin. To date, all cell lines that have been grown in soft agar, and successfully serially passaged a second time in soft agar, have produced tumors when injected in nude mice. No cell populations, after being blocked for 48 or 72 h and treated in S with Aflatoxin B_1-IN or MNNG-IN, formed colonies in soft agar or produced tumors in nude mice.

DISCUSSION

As previously reported (Milo and DiPaolo, 1978), we have successfully induced transformation of normal human cells with different chemical carcinogens. Other reports suggest that randomly proliferating normal skin-cell populations can be transformed by 4-nitroquinoline oxide and MNNG. However, we have found that successful induction of neoplastic transformation in rapidly proliferating normal human foreskin cells by chemical carcinogens *in vitro* is an exceedingly rare event. Chemicals that act as carcinogens in neonatal foreskin cells *in vitro* can damage cellular DNA (Milo *et al.*, 1978), but the DNA repair systems in these normal diploid cell populations are extremely rapid and error-free (Maher *et al.*, 1977).

Randomly proliferating normal cell populations repair over 90% of the damage from chemical carcinogens in 4-10 h (Milo and Hart, 1976) and are rarely transformed. There are two methods for synchronizing mammalian cells in the G_1 phase of the cell cycle: one is to arrest the cells in confluence and the other is amino acid deprivation (Peterson *et al.*, 1974; Jones *et al.*, 1976*b*, 1977; Greenberg *et al.*, 1978; Grisham *et al.*, 1979). Following release from the block nearly all the cells enter S phase within 6 to

TABLE II

FREQUENCY OF COLONY GROWTH IN SOFT AGAR OF TRANSFORMED HUMAN CELLS TREATED WITH DIFFERENT EXTRINSIC FACTORS AND AFLATOXIN B_1

Chemical (μg/ml)[1]	No. of treated populations	No. of lines that grew in agar / No. of lines seeded in agar	Frequency
Control (0)	0	0	0
Aflatoxin-B_1 (10)	10	3/20	$1:10^{5.2}$
Aflatoxin-B_1 (10) - IN (1)	10	10/10	$1:10^{5.1}$
IN (1)	3	2/3	$1:10^{5}$
Aflatoxin-B_1 (10) E_2 (1)	2	2/2	$1:10^{4.0}$
E_2	2	1/2	$1:10^{5.2}$
Aflatoxin-B_1 (10) C20:4 (1)	3	3/3	$1:10^{5.2}$
C20:4 (1)	3	1/3	$1:10^{5.4}$
Aflatoxin-B_1 (10) C18:1 (1)	3	1/3	$1:10^{5.7}$
C18:1 (1)	3	0/3	0
Aflatoxin-B_1 (10) d-cGMP (1)	2	1/2	0
d-cGMP (1)	2	0/2	0
Aflatoxin-B_1 (10) PMA (1)	5	5/5	$1:10^{4.7}$
PMA (1)	3	0/3	0
Aflatoxin-Anth (1)	4	4/4	$1:10^{4.5}$
Anth (1)	3	1/3	$1:10^{5.2}$

[1] The carcinogenic activity of Aflatoxin B_1 in cultures in the presence of added factors that altered the response pattern is presented. The exogenous factors selected for this study were insulin (IN); estradiol (E_2); arachidonic acid (C20:4); oleic acid (18:1); dibutyryl cyclic GMP (d-cGMP); phorbol myristate acetate (PMA); and anthralin (Anth). Each of these factors was added at point B as described in *Figure 5. Ten hours later, as the* cells were entering S, aflatoxin B_1 was added to the cells. After the treated cells had passed through S both the cultures treated with the factor alone and/or carcinogen and factor were removed (18.3 h) after point B (Fig. 1). *Column 1 presents the concentration of the carcinogen and factor added to* the cells. Column 2 presents the number of lines that grew in soft agar/number of lines seeded in soft agar. Column 3 presents the frequency obtained per number of lines that grew in soft agar. Frequency is *the number of colonies formed* in 21 days per 50,000 cells seeded into an 0.35% agar overlay (Milo and DiPaolo, 1978). The results were expressed as log values to the base 10.

TABLE III

EFFECT OF EXTRINSIC FACTORS ON GROWTH OF TRANSFORMED CELLS IN SOFT AGAR AND TUMOR FORMATION *IN THE NUDE MOUSE*

Treated cell populations	Extrinsic factors	Frequency of growth in soft agar[1]	Growth in nude mouse[2]	No. of successes / No. of attempts[3]
Aflatoxin B_1	None	+	+	1/8
	IN	+	+	7/7
	Anth	+	+	5/7
	PMA	+	+	2/8
	E_2	+	+	4/11
	C20:4	−	−	
	C18:1	−	−	
	HC	−	−	
	d-cGMP	−	−	
None	None	−	−	
MNNG	None	+	+	1/11
	IN	+	+	4/5
	Anth	+	+	5/5
	PMA	+	+	3/9
	E_2	+	+	2/8
	C20:4	−	−	
	C18:1	−	−	
	HC	−	−	
	d-cGMP	−	−	
None	None	−	−	

Column one identifies the carcinogen used to treat each of the transformable cell populations. The *extrinsic factors* selected for this study were either insulin (IN); estradiol (E_2); anthralin (Anth); phorbol myristate acetate (PMA); arachidonic acid (C20:4); oleic acid (C18:1); hydro-cortisone (HC); or dibutyryl-cyclic GMP (d-cGMP). [1] The frequency of growth in soft agar represents *the number* of lines that grew in soft agar. Plus (+) represents 100% of lines that were seeded produced colonies 14–21 days after seeding into 0.33% soft agar over a 5 ml 2.0% agar base (see text for details). Minus (−) none of the lines seeded grew in soft agar. *Minimum number of lines* seeded was 5. [2] Transformed cells from all (+) lines were injected at a cell concentration of 5×10^6 cells/0.5 ml into the subscapular region of the irradiated nude mouse. The tumors were allowed to grow to 0.6–1.2 cm in size (Milo and DiPaolo, 1978). *Non-transformed cell lines* (−) did not form tumors. [3] This column represents the number of mice that produced tumors 4 weeks after injection of the cell pack per total number of mice injected for a single transformed cell line isolated from agar. Repeat experiments with other *transformed cell lines* yielded similar results (4 other lines per treatment). Control (untreated cells) inocula did not grow in soft agar and when injected directly from culture (10^7 cells per inoculum) into the mouse the bleb(s) regressed in 24 hours.

8 h. C_3H 10T1/2 cells arrested in this manner, released and treated with MNNG in S phase, exhibit an increase in sensitivity to MNNG and become more readily transformed. However, isoleucine-deficient medium does not block human cells in G_1. Furthermore, release of human cells from block by cell subpassaging, after density-dependent inhibition in G_1 with carcinogens over a 10-h period, does not result in a carcinogen-induced transformation event. In fact, the transformation of human cells at early passage levels is inhibited if the transformable cells are kept in a density-inhibited state for 8-16 h prior to subpassaging. Moreover, cultures held in the G_1 phase of the cell cycle for more than 24 h cannot be transformed when treated with aflatoxin B_1 or MNNG in the S phase. A double amino-acid-deficient medium (minus glutamine and arginine) was necessary to adequately block the cells in G_1. Augmentation of the transformation occurs when IN, Anth, E_2, or PMA are added to the cultures prior to carcinogen administration. The major difference between the effectiveness of aflatoxin B_1 and MNNG as carcinogens appears to be the time at which the compounds are added to the cells entering S. MNNG is more effective if added early in S (0–4 h after recovery from the amino acid block) while aflatoxin B_1 is more effective if added from 2 h before S to 4 h into S. The incidence of transformation was reduced to less than 1 out of 10 when these compounds were added to the cells in late S (4 to 8 h into S).

IN, PMA, E_2, and Anth modulate the expression of other transforming agents, and the effects on chemical carcinogen-induced carcinogenesis are not unique. Enhancement of virus-induced transformation by some of the same chemical factors used here occur with E_2 (Milo *et al.*, 1972), HC (Schaller *et al.*, 1976), and PMA (Weinstein *et al.*, 1979), these chemicals can interfere with semiconservative DNA synthesis (Milo and Hart, 1975). Modulation of cellular functions is not an unusual response by cells to the presence of these compounds and they obviously facilitate transformation by chemical carcinogens. This suggests that, as in the 3T3 system described by Grisham *et al.* (1979), human cell subpopulations are sensitized in S to carcinogens by amino-acid deprivation and reconstitution. This sensitization is enhanced by Anth, IN, PMA or E_2 at pharmacological concentrations of the drugs.

Many of the compounds studied here (E_2, HC, C20:3, C20:4, IN) are components of fetal bovine serum and serve as natural regulatory agents of proliferation; the action of PMA has been suggested to resemble that of hormonal agents. The pleiotypic responses of subpopulations to the carcinogen treatment may be induced by pretreatment with PMA, E_2, Anth or IN. Therefore, after subsets of populations have been modulated by being kept in G_1 for 48 h or serially subpassaged *in vitro* to PDL >5, the cells no longer respond to the extrinsic factors in the presence of the carcinogens.

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REFERENCES

BASERGA, R., Autoradiography. *In:* H. Busch (ed.) *Methods in cancer research*, Vol. 2, pp. 45-116. Academic Press, New York (1967).

CRISTOFALO, V., and SHARF, B., Cellular senescence and DNA synthesis: thymidine incorporation as a measure of population age of human diploid cells. *Exp. Cell Res.*, **76**, 419–427 (1973).

GREENBERG, D., GRISHAM, J., BELL, W., BAKER, M., and KAUFMAN, D., Differing effects of isoleucine deficiency on toxicity of MNNG for 10T1/2 and CHO cell. *In Vitro*, **14**, 516-521 (1978).

GRISHAM, J., GREENBERG, D., SMITH, G., and KAUFMAN, D., Temporary culture in isoleucine-free medium enhances transformation of 10T1/2 cells by N-methyl-N'-nitrosoguanidine (MNNG). *Biochem. Biophys. Res. Comm.*, **87**, 969-975 (1979).

GROVE, G., and CRISTOFALO, V., Characterization of the cell cycle of cultured human diploid cells: effects of aging and hydrocortisone. *J. Cell Physiol.*, **90**, 415-422 (1977).

HART, R., and SETLOW, R., DNA repair in late passage human cells. *Mech. Aging Develop.*, **5**, 67-77 (1976).

HUTTNER, J., MILO, G., PANGANAMALA, R., and CORNWELL, D., Fatty acids and the selective alteration of *in vitro* proliferation in human fibroblasts and guinea-pig smooth-muscle cells. *In Vitro*, **14**, 854–859 (1978).

JONES, P., BENEDICT, W., BAKER, M., MONDAL, S., RAPP, U., BERTRAM, J., and BENEDICT, W., Cell cycle-specific oncogenic transformation of C_3H/10T1/2 clone 8 mouse embryo cells by 1-B-D-arabinofuranosylcytosine. *Cancer Res.*, **37**, 2214-2217 (1977).

JONES, P., BENEDICT, W., BAKER, M., MONDAL, M., RAPP, U., and HEIDELBERGER, C., Oncogenic transformation of C_3H/ 10T1/2 clone 8 mouse embryo cells by halogenated pyrimidine nucleosides. *Cancer Res.*, **36**, 101-107 (1976).

MAHER, V., MCCORMICK, J., GROVER, P., and SIMS, P., Effect of DNA repair on the cytotoxicity and mutagenicity of polycyclic hydrocarbon derivatives in normal and *Xeroderma pigmentosum* human fibroblasts. *Mutation Res.*, **43**, 117-138 (1977).

MERZ, G., and ROSS, J., Clone size variation in the human diploid cell strain, WI-38. *J. Cell Physiol.*, **82**, 75-80 (1973).

MILO, G., ACKERMAN, A.G., and NOYES, I., Growth and ultrastructural characterization of proliferating human keratinocytes *in vitro* without added extrinsic factors. *In Vitro*, **16**, 1-11 (1980).

MILO, G., and DIPAOLO, J.A., Neoplastic transformation of human diploid cell *in vitro* after chemical carcinogen treatment. *Nature (Lond.)*, **275**, 130-132 (1978).

MILO, G., and HART, R., Effects of steroid hormones on semiconservative and UV-induced unscheduled DNA synthesis. *In Vitro*, **10**, 346 (1975).

MILO, G., and HART, R., Age-related alterations in plasma membrane glycoprotein content and scheduled or unscheduled DNA synthesis. *Arch. Biochem. Biophys.*, **176**, 110-118 (1976).

MILO, G., MALARKEY, W., POWELL, J., BLAKESLEE, J., and YOHN, D., The effects of steroid hormones in fetal calf serum on plating and cloning of human cells *in vitro*. *In Vitro*, **12**, 23-30 (1976).

MILO, G., YOHN, D., and SCHALLER, J. Hormonal modification of adenovirus transformation of hamster cells *in vitro*. *Cancer Res.* **32**, 2338–2347 (1972).

PETERSON, A., BERTRAM, J., and HEIDELBERGER, C. Cell cycle dependency of DNA damage and repair in transformable mouse fibroblasts treated with N-methyl-N′-nitro-N-nitrosoguanidine. *Cancer Res.* **34**, 1600–1607 (1974).

RIEGNER, D., MCMICHAEL, T., BERNO, T., and MILO, G. Processing of human tissue to establish primary cultures *in vitro*. *Tissue Culture Association Laboratory Manual*. Vol. 2, pp. 273–276 (1976).

SCHALLER, J., MILO, G., BLAKESLEE, J., YOHN, D., and OLSEN, R. Influence of glucocorticoid, estrogen and androgen steroids. *Cancer Res.* **36**, 1980–1987 (1976).

TOBEY, R., and LEY, K. Isoleucine-mediated regulation of genome replication in various mammalian cell lines. *Cancer Res.* **31**, 46–51 (1971).

TURK, B., and MILO, G. An *in vitro* study of senescent events of human embryonic lung (WI-38) cells. I. Changes in enzyme activities of cellular and membrane associated enzymes of untreated and cortisone acetate treated cultures during senescence. *Arch. Biochem. Biophys.* **16**, 46–53 (1974).

WEINSTEIN, I. B., YAMASAKI, H., WIGLER, M., LEE, L-S., FISHER, P., JEFFEREY, A., and GRUNBERGER, A. Molecular and cellular events associated with the action of initiating carcinogens and tumor promoters in carcinogens. *In* C. Griffin and C. Shaw (eds.), *Carcinogens: Identification and mechanisms of action*. Raven Press, New York (1979).

IN VITRO
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GROWTH AND ULTRASTRUCTURAL CHARACTERIZATION OF PROLIFERATING HUMAN KERATINOCYTES IN VITRO WITHOUT ADDED EXTRINSIC FACTORS[1]

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SUMMARY

Routine in vitro cultivation of human epithelial cells derived from foreskin and free of contaminating fibroblasts has been achieved without the addition of conditioned medium or extrinsic factors. Epithelial cell populations could be serially subpassaged and exhibited modulating responses at PDL5 to culture conditions as the cells passed from phase 1 through phase 2 of their life span. The cell population in early phase 2 gave rise to tissue sheets that exhibited characteristics typical of human foreskin epidermis including the formation of distinct cellular layers, viz. strata basalis, spinosum, granulosum and corneum. Typical keratohyaline granules were not observed in the epithelial cells although a distinct cornified layer was evident. Ultrastructurally, desmosomes and tonofilaments were readily apparent. Thus, the procedure detailed in this study will produce highly differentiated fibroblast-free epidermal sheets reaching several centimeters in size and which can be removed from the substratum as a single sheet of organized epidermis. The epithelial cells could be cultured through 20 ± 3 PDL, whereas fibroblast cultures derived from foreskin cultures exhibited 40 ± 5 PDL and mixed cell cultures of foreskin were carried through 43 ± 5 PDL.

Key words: epithelial cells; human skin cultures; skin epithelial cells; epidermis culture; ultrastructure of cultured epithelial cells.

INTRODUCTION

Reproducible in vitro cultivation of normal proliferating human epithelial cells has been difficult to achieve with present methodologies and the procedures developed to establish primary epithelial cell populations have resulted in a low rate of success (1–4). Enhanced establishment of epithelial cell populations from explants (5,6) occurs with the addition of fibroblasts or extrinsic growth factors, i.e. products released from cultured fibroblasts (conditioned growth media) (7), epidermal growth factor (3) or hydrocortisone (8), to the cultures or culture media. Recently, Freeman et al. (9) reported that by the use of a dermal collagen bed derived from sterile pig skin, human epithelial cell cultures were established in 129 of 140 attempts; the cultured epithelial cells grew in the absence of fibroblasts or their products (except collagen). Earlier, we described a method for the enzymatic dispersion, growth and serial subpassage of primary cultures of human fibroblasts derived from foreskin (10); epithelial colonies were occasionally noted in these cultures after subpassage. We have now modified the culture technique in a manner that permits not only the establishment of pure populations of human fibroblasts but, more importantly, allows for the successful establishment of pure human epidermal epithelial cell cultures. The human epithelial cells were grown successfully without the addition of extrinsic growth factors or collagen substrata and have

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been serially subpassed, fibroblastfree, on a routine basis. Epithelial cells could be grown to form large multilayered epidermal-like sheets in which the cells showed differentiative characteristics consistent with those noted in normal intact epidermis for a limited number of PDL (PDL 5).

It is the purpose of this report to detail the methodology used for the routine culture of human foreskin epithelial cells (keratinocytes), which grow to form large multilayer cell sheets. The growth characteristics and morphology of the cultured cells also will be described.

Materials and Methods

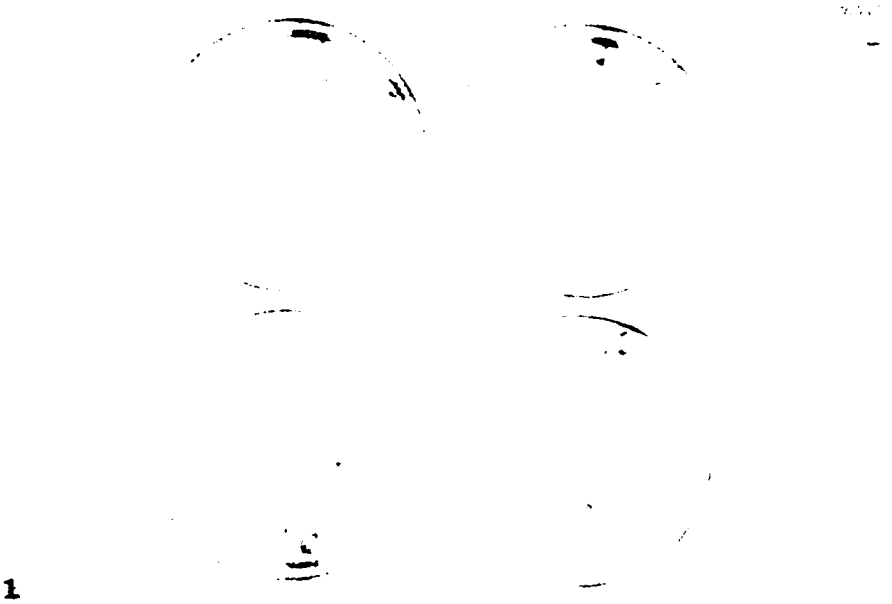
Preparation of cell suspensions. Human foreskin was obtained from infants at the time of circumcision. The epidermis was dissected carefully from the underlying connective tissue dermis and cut into 2-mm pieces in MEM-Hanks' balanced salt medium containing 25 mM HEPES buffer at pH 7.2 (CM) culture medium). The tissue was rinsed three times in this medium and the tissue fragments transferred to 20 ml of CM medium supplemented with 20% fetal bovine serum (FBS) containing 0.25% collagenase (115 U per mg, 4197 CLS, Worthington Biochemical Corp., Freehold, New Jersey). Enzymatic tissue dispersion was done at 37° C in a 4% CO_2-enriched air atmosphere for 5 hr or overnight. Cells were recovered from suspension by centrifugation at 150 × *g* for 7 min at 4° C. The cell pellet was washed twice with CM medium and seeded into 75-cm² flasks. After seeding, the cell cultures had to be refed at 48 hr with 15 ml of the CM medium supplemented with 20% FBS. Three to five days later, cultures were observed for the appearance of epithelial colonies, and the mixed cell cultures were allowed to grow to confluency. It should be noted that epithelial cell growth was dramatically inhibited by addition of either penicillin, streptomycin, aureomycin or fungizone. Therefore, antibiotics were not added to the culture medium.

Preparation of epithelial cell cultures. At confluent density or when cultures reached a diameter of 5 to 9 mm, primary mixed cell cultures were trypsinized in order to remove fibroblasts. The longer the cultures were left in confluent density, the more difficult it became to selectively remove the fibroblast population: 16 hr after the cultures reached confluency proved an optimum time to do this. CM was removed from the mixed cell cultures and the cultures rinsed twice. One milliliter 0.1% trypsin (Worthington Biochemical Corp., lypholized 9300 BAEE U per mg, lot TL-3BP) in CM was layered over the culture monolayers.

After 90 seconds, the fibroblasts floated off the substratum while the epithelial sheet remained firmly attached to the substratum. The enzymatic action was stopped by the addition of 10% FBS-supplemented CM. Residual fibroblasts were removed by rinsing the flask twice with growth medium. These fibroblasts were seeded in separate culture vessels and subpassed as previously described (10). The epithelial cultures were fed with 11.5 ml CM medium, 1.5 ml FBS and 3 ml minimum essential vitamin mixture (100x concentrated) [CM plus vitamin supplemented (CM-V), Microbiological Associates, Walkersville, Maryland]. The reduction in supplementation of FBS from 20 to 10% or even 5% diminishes the growth rate of residual fibroblasts while not adversely affecting the growth of the epithelial cells. Trypsinization was repeated 2 to 4 times at 3-day intervals. Epithelial cultures were allowed to grow for at least 2 weeks and were refed every 4 days with CM-V.

Serial subpassage of epithelial cells. Subpassage was initiated within 2 to 4 weeks after seeding of the primary cultures. In preparation for subpassage, the CM-V medium was decanted and the epithelial cell sheet rinsed with 10 ml of Mg^{++}-, Ca^{++}-free MEM containing 0.02% tetra sodium ethylene diaminetetraacetate (EDTA; Eastman Kodak, Rochester, New York) at pH 7.2. Cultures were then treated with a 0.1% trypsin solution in Mg^{++}- Ca^{++}-free MEM, 0.02% EDTA, for 90 seconds. Trypsin activity was neutralized by addition of 15 ml of CM-V medium containing 10% FBS. The epithelial colonies lost continuity and individual cells became detached from the substratum. The dish (or flask) was gently shaken increasing detachment: free cells floated in the medium.

The free-floating epithelial cells were recovered by centrifugation at 150 × *g* for 7 min, and the cell pellet was resuspended in CM-V medium supplemented with 10% FBS. After rinsing the pellet once in CM-V medium, the suspended cells were seeded into 25-cm² flasks or plates at a cell density of 50,000 cells per cm². Subsequent subpassages were done in a similar manner. Within 15 to 20 min after seeding, the cultures were gently rotated by hand for about 5 min to encourage aggregation of the single cells before attachment to the substratum: without this step a noticeable decrease in the numbers of subsequent epithelial colonies was observed.



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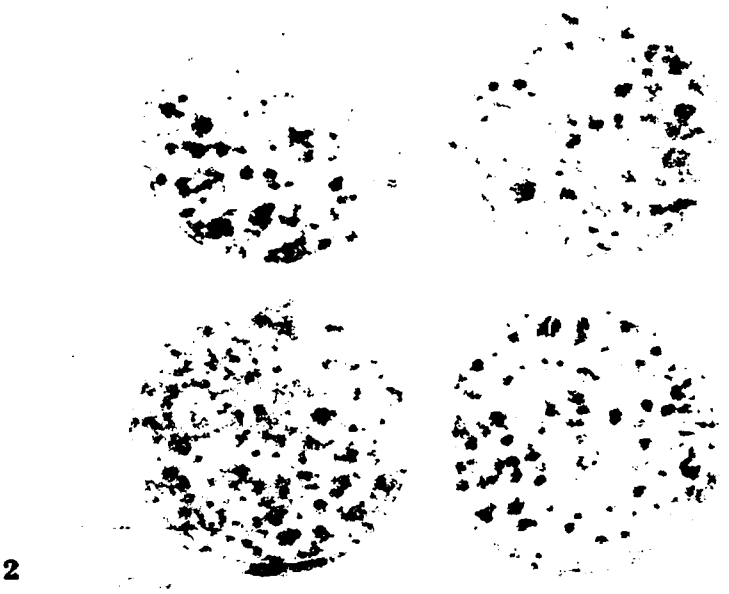

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FIG. 1. Colonies of epithelial cells after 3 weeks after seeding 500 cells per dish (25 cm²), buffered with formalin and stained with hematoxylin eosin. ×½.

FIG. 2. Colonies of human fibroblast seeded at 1000 cells per dish (25 cm²) from PDL 2, fixed in phosphate buffered formalin and stained with hematoxylin eosin. ×½.

TABLE I

GROWTH CHARACTERISTICS OF FIBROBLAST EPITHELIAL AND MIXED CELL POPULATIONS IN VITRO

Culture Type	Cells seeded (75 cm²)	Cell Density at 7 Days (75 cm²)	PDL Days After 1:4 Split	Life Span PDL
Fibroblast	375,000	$1.5 - 2 \times 10^6$	3	40 ± 5
Epithelial[a]	250,000	$4 - 6 \times 10^6$	14	20 ± 3
Mixed	25,000	2×10^6	3	43 ± 5

[a] These cell populations exhibit alterations in morphology as they are serially subpassaged.

Preparation of epithelial and fibroblast cultures for microscopy. Cell cultures examined for their growth patterns and morphology in culture were fixed in formalin and stained with hematoxylin. For electron microscopy, epithelial cultures were washed with CM media and exposed to 0.1% collagenase in 10% FBS-supplemented growth medium at 37° C in 4% CO_2-enriched air atmosphere for 4 to 12 hr in order to free the cells or colonies from the substratum. These cell sheets (2 to 50 cm² in area) were removed and fixed in 3% glutaraldehyde in 0.15 *M* cacodylate buffer, pH 7.4, for 30 min at room temperature or overnight at 4° C. Confluent cultures of fibroblasts were scraped into sheets and fixed in 3% glutaraldehyde. Cell preparations were subsequently post-osmicated in 1% chrome-osmium tetroxide for 1 hr at 4° C, dehydrated and embedded in Araldite. Thick (1 μm) sections were stained with 1% basic fuchsin in 50% acetone or with aqueous 0.1% toluidine blue for light microscopy. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate.

RESULTS

Growth characteristics of epithelial and fibroblast populations. Epithelial cultures, freed of fibroblasts, grew in discrete colonies (Fig. 1). Cell colonies at confluency formed large continuous sheets (75 cm²) in size. On occasion, the cell sheet would spread up the side of the well or around the neck of the culture flask, a feature never observed with fibroblast cultures. Optimal cell density for seeding of epithelial cultures was found to be 50,000 cells per cm² and a population doubling occurred after 14 days (11). Pure epithelial cultures (PDL 2 to 5) exhibited cellular stratification rather than forming true monolayers; cells were always found to be in contact or intimately attached with the adjacent cells of the patch or

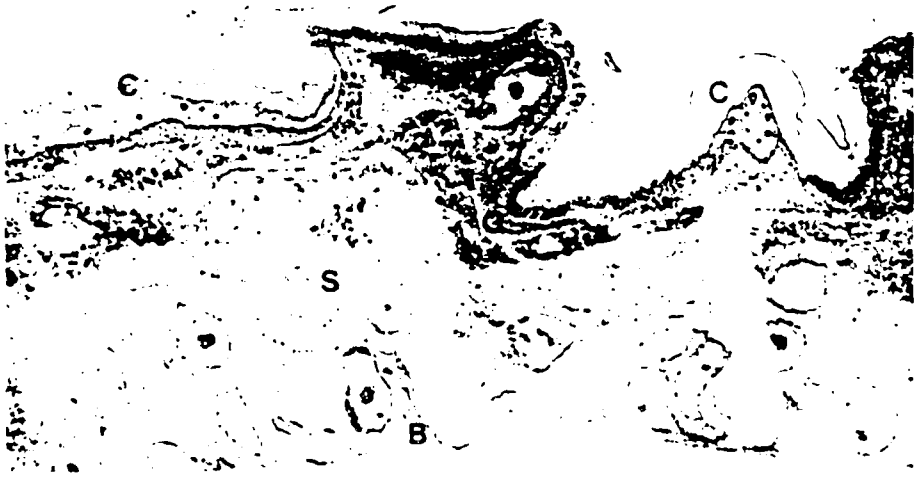

FIG. 3. Transverse section through the more central region of an epithelial sheet from cultures at PDL 1. Note stratification of the epithelial cells: B: stratum basalis, S: stratum spinosum, G: stratum corneum. C. 1-μm Araldite section stained with basic fuchsin. ×500.

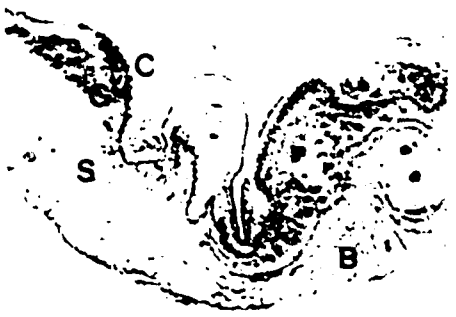

FIG. 4. Transverse section through the marginal region of the epithelial sheet from cultures at PDL 1. Note that the number of cell layers and thickness of this area is less than in Fig. 1. Individual strata can be seen. Stratum basalis, B; stratum spinosum, S; stratum granulosum, G; stratum corneum, C. 1-um Araldite section stained with basic fuchsin. ×700.

sheet. These growth characteristics were maintained for five PDL after which distinctive changes occurred in the growth pattern that will be detailed in a subsequent paper. Fibroblast populations removed from the mixed foreskin cultures and seeded on a separate substratum exhibited typical fibroblast growth patterns (Fig. 2), growing in definite parallel whorl-like patterns, which were of variable size; cells were not attached to one another. The fibroblast growth characteristics were maintained for PDL 40 (Table 1) when cloned from 100 cells per cm^2 or at a high density of 5,000 cells per cm^2. Saturation growth density of subcultures of fibroblasts decreased from 20,000 cells per cm^2 (PDL 1 to 15) (early phase 2) to 15,000 cells per cm^2 (PDL 16 to 31) (middle phase 2), and cultures would not reach a confluent state after PDL 32 (Table 1) (late phase 2).

Microscopy of the epithelial cell population. Light microscopic examination of the epithelial colonies demonstrated their stratified nature (PDL 1 to 5). The central region of the colonies was 6 to 8 cells in thickness (Fig. 3), whereas the marginal zone was much thinner and consisted of 3 to 5 cell layers (Fig. 4).

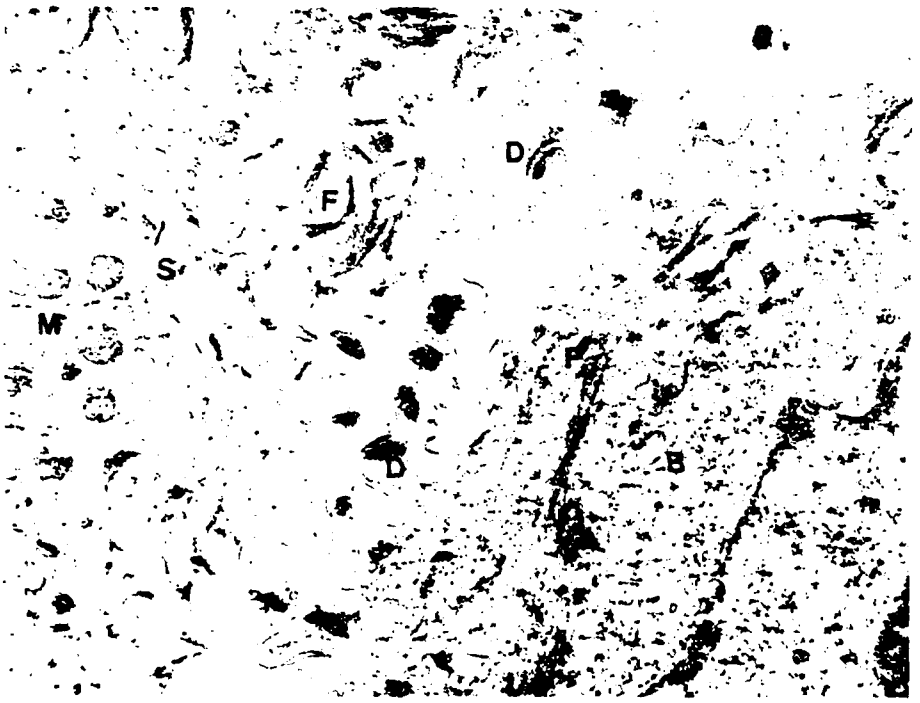

FIG. 5. Epithelial cells of the basal (B) and spinosum (S) layers. Note filament (F) bundles and desmosomes (D). Several mitochondria (M) are also indicated. Stained with uranyl acetate and lead citrate. ×27,000.

FIG. 6. Epithelial cells from the upper portion of the spinosum. Cytoplasmic filaments (F) and desmosomes (D) are prominent. Small cytoplasmic granules (O) are indicated near the plasma membrane. Stained with uranyl acetate and lead citrate. ×50,000.

The structural organization of epithelial sheets was strikingly similar to that of typical foreskin epidermis, and distinct cell layers or strata as seen in the epidermis were readily identified, viz. strata basalis, spinosum, granulosum and corneum (Figs. 3,4). The stratum spinosum of the epithelial sheets was generally 2 to 3 cells thick and rested on a single row of basal cells, which formed the stratum basalis. The epidermal cells of the stratum spinosum were polygonal in contour, featuring abundant cytoplasmic filaments. Junctional zones were evident between cytoplasmic processes of adjacent cells. Prominent cytoplasmic granules, which stained intensely with basic fuchsin and with toluidine blue, characterized the flattened cells comprising the stratum granulosum; this stratum varied from one to two cells in thickness. The most superficial cell layer, the stratum corneum, was one to two cells in thickness. These cells were quite flat, lacked nuclei and their cytoplasm appeared amorphous and stained poorly; granules were not visible in the cytoplasm. The surface of the cornified cells in direct contact with the culture medium appeared thickened and was darkly stained. In some instances, the superficial cells of the epithelial sheet were partially separated from the underlying cells. Fibroblasts were not observed either by light or electron microscopy in epithelial sheet cultures.

Ultrastructurally, the cells of the strata basalis and spinosum exhibited extensive arrays of filament (tonofilaments) bundles in their cytoplasm; desmosomes that joined adjacent epithelial cells were common (Figs. 5,6). Epithelial cells from these cell zones had numerous mitochondria, scattered profiles of rough endoplasmic reticulum and Golgi complexes of modest size. The number of polysomes and the electron density of the cytoplasmic matrix were generally greater in basal cells than in the cells of the stratum spinosum. Epithelial cells comprising the stratum granulosum (Figs. 7,8) varied moderately in their ultrastructure. Cytoplasmic filaments of these cells were less clustered than noted in the stratum spinosum, and the filaments tended to be aligned along the long axis of the cell. Desmosomes were common but filament attachment to these structures were less evident than seen in the stratum spinosum. The discrete cytoplasmic granules evident by light microscopy in the granulosal cells were quite conspicuous at the electron microscopic level. These structures appeared as large, irregularly shaped, membrane-bound granules (Figs. 8–10), which varied in size, electron density and internal structure. Small membrane-bound vesicles and myelin figures (Fig. 7) were commonly found within these granular structures, which indicates morphologically their similarity to secondary lysosomes and lipofuchsin granules, or both. Transitional stages in the formation of these lysosomal-like granules were noted in some granular cells and occasionally in cells of the stratum spinosum, which were in direct apposition with the cells of the stratum granulosum

(Fig. 9). Typical keratohyaline granules were not observed in the epidermal cells although present in the native foreskin epidermis. Relatively few mitochondria were present in the epithelial cells of the stratum granulosum and nuclei were uncommon in this layer of the cultured epithelial sheets. Small electron-dense, round-to-oval, membrane-bound granules with electron lucent clefts or zones (Figs. 6,7) provided an additional morphological feature of the granular cells and cells of the stratum spinosum in immediate apposition to the stratum granulosum. It is noteworthy that these small granules concentrated along the plasma membrane on the side of the cell directed toward the stratum corneum. These small granules closely resemble morphologically, and by position, the mucus-coating granules (MCG) described in normal epidermis.

The most superficial layer of the epidermal sheet (Figs. 7, 10) resembled the typical stratum corneum of foreskin epidermis, although this stratum was only one to two cells thick in the culture preparations. The cells were quite flattened and lacked nuclei and cell organelles. Tonofilaments were abundant, oriented parallel to the long axis of the cell and embedded in an amorphous material of low electron density (Fig. 10). The plasma membrane was thickened when compared to the cell membranes of other cells of the epidermal sheet. Modified desmosomes, similar to the modified desmosomes described for normal epidermis, were evident between the cornified

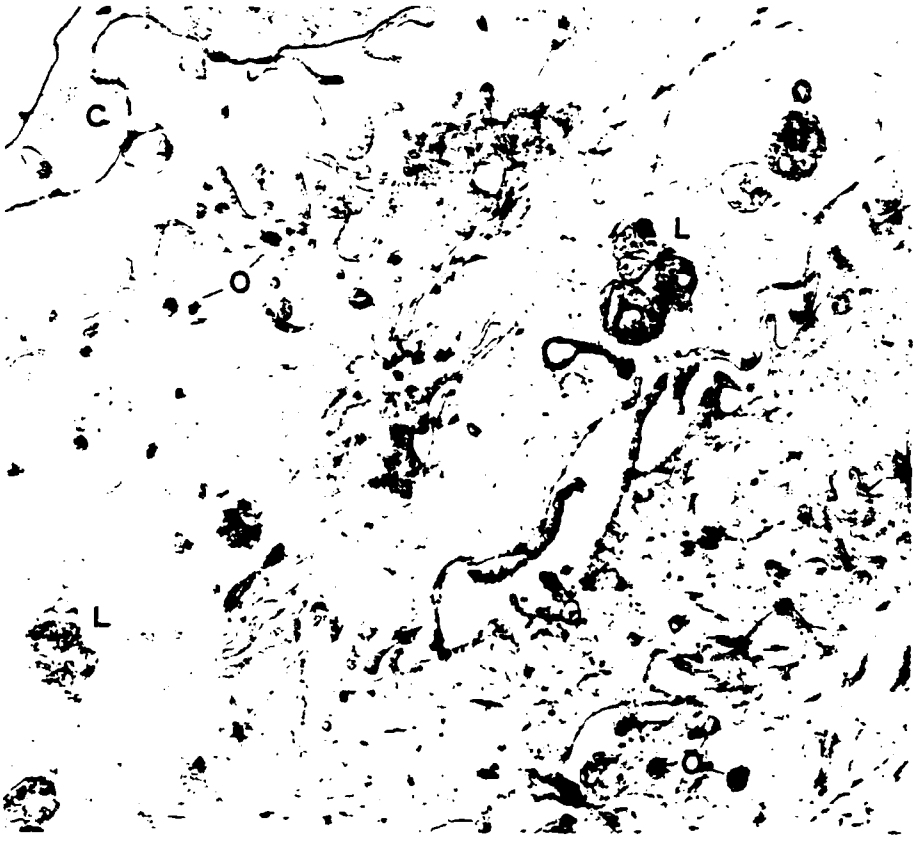

FIG. 7. Superficial portion of the epithelial sheet shows a cornified cell (C) and several cells of the stratum granulosum. Secondary lysosomes or lipofuchsin granules (L) are evident. In addition, note the small granules (O) concentrating near the plasma membrane. Stained with uranyl acetate and lead citrate. ×21,000.

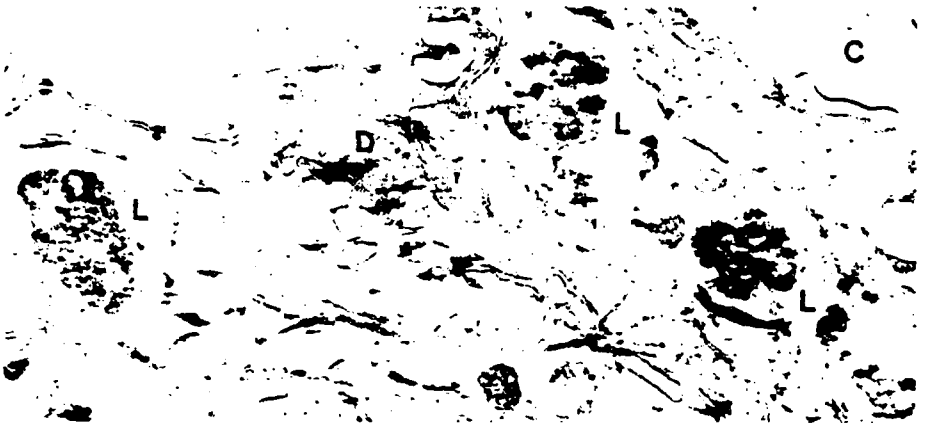

FIG. 8. Higher magnification of the secondary lysosomes or lipofuchsin granules (L) of granular cell in the stratum granulosum demonstrates their variable internal structure. Desmosomes are shown at *D* and a portion of an epithelial cell (C) of the stratum corneum. Stained with uranyl acetate and lead citrate. ×27,000.

(keratinized) cells. Transitional forms between typical granulosal cells and the superficial cornified cells were found.

Morphology of the fibroblast population. Fibroblast cultures derived from the mixed cultures of foreskin appeared quite distinct (Fig. 11) from the cells of the epithelial colonies and sheets. Most of these cells occurred singly. Cells in contact with one another were not attached by desmosomes. Fibroblasts contained both smooth and

FIG. 9. Epithelial cell located at the junction of the stratum spinosum and granulosum. Note morphological stages in the formation of the large secondary lysosomes or lipofuchsin granules (L). Cytoplasmic filaments (F) and mitochondria (M) are indicated. Stained with uranyl acetate and lead citrate. ×44,000.

rough endoplasmic reticulum. The rough endoplasmic reticulum was frequently distended and such profiles had few attached ribosomes. Mitochondria were numerous and glycogen was abundant; autophagic vacuoles were present. At higher magnifications, cytoplasmic filaments were evident but were less numerous than in the epithelial cell and did not form discrete bundles as seen in the epithelial cell cultures.

Discussion

We have been able to culture and serially subpass epithelial cells derived from normal and human foreskin. Pure epithelial cultures free of contaminating fibroblasts were obtained and maintained in typical epithelial-like cultures through five PDL. Epithelial growth patterns were distinctive, and cell colonies, when grown to confluency, formed large sheets several layers in thickness with adjacent cells joined by desmosomal junctions. Self-limiting islands of epithelial cells surrounded by fibroblasts, noted by others using alternative procedures to prepare epithelial cell cultures in vitro (7,9,12-15), were not observed in this study. Cells comprising the epithelial sheets exhibited differentiative changes identical to those occurring in normal epidermis of the intact foreskin. Distinctive cell strata were observed in the epithelial cultures. Epithelial cells or keratinocytes possessed tonofilaments, desmosomal junctions and mucus-coating granules. Thickened cell membranes of the enucleated superficial cornified cells plus modified desmosomes and organized tonofilaments in an amorphous matrix characterized these fully differentiated surface epithelial cells (19). Differentiative changes in each cell strata of the cell sheets were identical with those seen in normal epidermis except that keratohyaline granules (20) were absent in the stratum granulosum; however, secondary lysosomes or lipofuchsin granules were a conspicuous feature of the cells of the stratum granulosum of the epithelial cultures. The absence of keratohyaline granules may reflect the cell's inability to synthesize these granules while rapidly

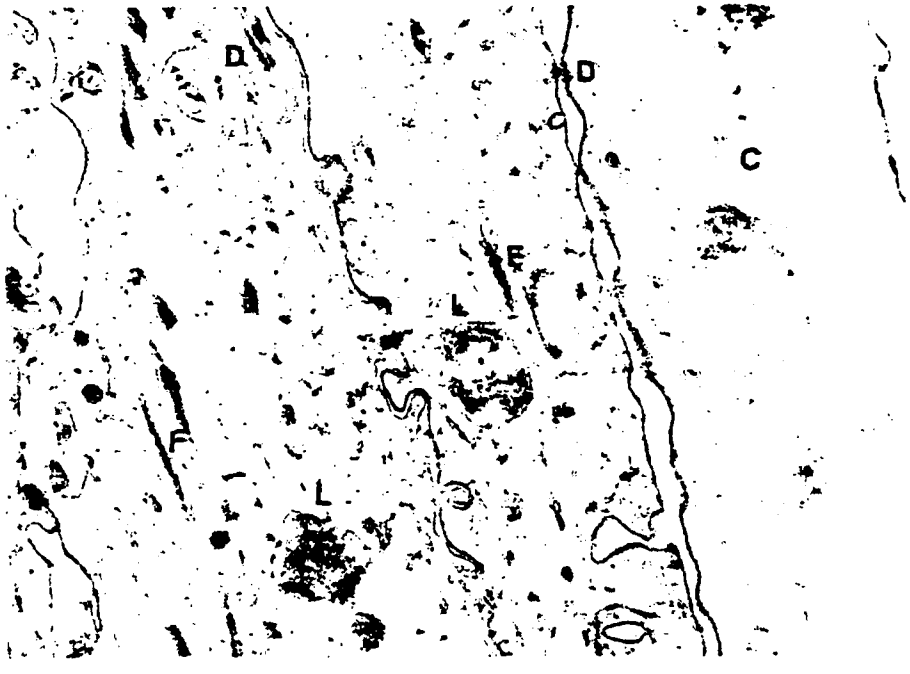

FIG. 10. Note the tonofilaments (F) have assumed an orientation along the long axis of the cells of the stratum granulosum. Desmosomes (D) and secondary lysosomes (L) are evident. The plasma membrane of cornified cell (C) is thickened and fine filaments are embedded in an amorphous matrix in this superficial cell of the epithelial sheet. Stained with uranyl acetate and lead citrate. ×29,000.

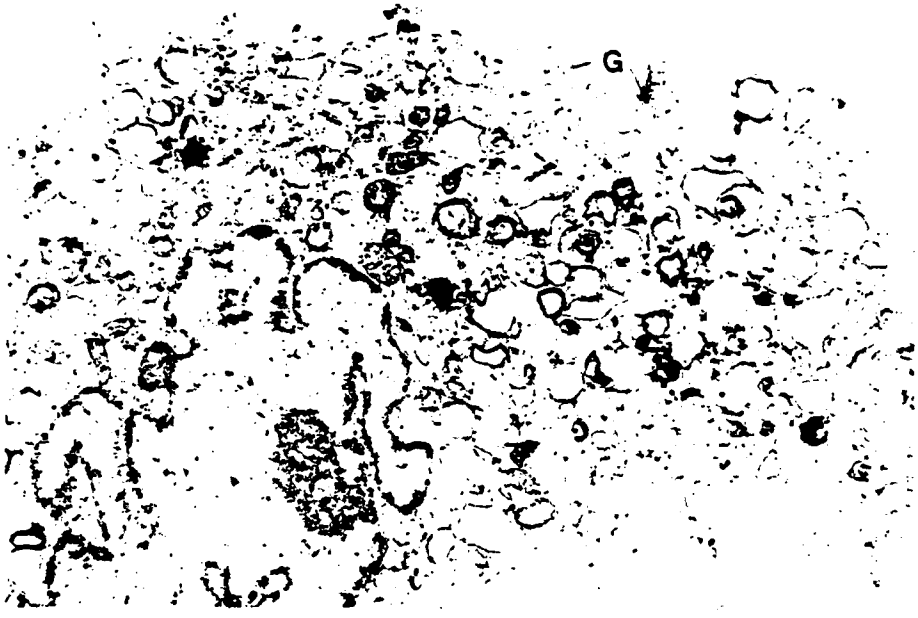

FIG. 11. Cultured fibroblasts derived from the mixed cultures are morphologically distinct from the cells of the epithelial sheets. There are no desmosomes and few filaments can be distinguished. Mitochondria are numerous, scattered dilated cisternae are evident and a number of small vacuoles are present in the cytoplasm; glycogen (G) is a common feature of these cells. Stained with uranyl acetate and lead citrate. ×17,000.

proliferating or because of in vitro culture conditions.

Variable degrees of success have been reported in the propagation of normal human epithelial cells, particularly those from human epidermis or skin. Explant culture of human epidermis commonly exhibited fibroblastic growth in association with the epithelial outgrowths suggesting that fibroblast interaction was necessary for achieving epithelial propagation and differentiation (16). In the system described here the presence of fibroblasts does not enhance growth of the epithelial cell population. On the contrary, fibroblasts, when left in mixed cell cultures, overgrow the epithelial cells, thereby inhibiting epithelial growth. After contaminating fibroblasts from mixed primary cultures of human foreskin were removed by selective trypsinization, we were able to subpassage the pure epithelial cell population up to five PDL without morphological alteration. Others (17) were able to subpass normal adult skin through four subpassages with modest differentiation evident in the culture strata.

Growth of human cell populations can be affected by the composition of the fetal bovine serum (15). Traditionally, we characterize (18) the fetal bovine serum prior to use on human cell populations. In addition, we have found that several types of antibiotics inhibited the establishment of proliferating epithelial cell cultures. We have found that the growth characteristics, cell attachments, proliferative characteristics and life span of the cultured epithelial cells from human foreskin were finite and were markedly different from cell cultures arising from a mixed cell population. Fibroblast cultures, derived from infant foreskin subcultures, when grown to confluency, ceased to grow except at terminal points of the whorling patterns; overlapping of cells only occurred at these sites. In contrast, epithelial cells grew in concentric ring patterns and were several cells in thickness.

Preliminary comparison of growth characteristics, as noted in Table 1, indicated that the life spans of epithelial cell populations were different in extent of proliferation from fibroblast PDL.

Moreover, the epithelial cell populations did exhibit characteristic senescent features in vitro. These cell populations, like the fibroblasts, passed through phases 1 and 2 (11) as seen by Karasek and Liu (19). They did not exhibit saturation density-dependent inhibition. The piling up of the epithelial cells into strata (20,21) may account for the increase in numbers of cells observed in the epithelial cultures. We have found that foreskins from adults can produce a ready source of keratinocyte cells that can be grown in vitro using the method described here. These epithelial cell populations also exhibit the growth characteristics associated with tissue in phase 2 and differentiative structures similar in anatomical characteristics to skin epidermis. This differentiative tissue produced in vitro does not require added extrinsic factors, such as epidermal growth factor, pituitary extract, conditional growth medium, hydrocortisone or the presence of collagen to restrict the proliferation of fibroblasts.

REFERENCES

1. Rafferty, K. A. Jr. 1973. Epithelial cells: Growth in culture of normal and neoplastic forms. Adv. Cancer Res. 21: 219–272.
2. Owens, R. B., H. S. Smith, W. A. Nelson-Rees, and E. L. Springer. 1976. Epithelial cell cultures from normal and cancerous human tissues. J. Natl. Cancer Inst. 56: 843–847.
3. Rheinwald, J., and H. Green. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature 265: 421–424.
4. Lake, R. S., M. L. Kropko, M. R. Pezzutti, R. H. Shoemaker, and H. J. Igel. 1978. Chemical induction of unscheduled DNA synthesis in human skin epithelial cell cultures. Cancer Res. 38: 2091–2098.
5. Flaxman, B., M. Lutzner, and E. J. Van Scott. 1967. Cell maturation and tissue organization in epithelial outgrowths from skin and buccal mucosa *in vitro*. J. Invest. Dermatol. 49: 322–330.
6. Constable, H. 1972. Ultrastructure of adult epidermal cells in monolayer culture. Br. J. Dermatol. 86: 27–39.
7. Rheinwald, J. G., and H. Green. 1975. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell 6: 317–330.
8. Rheinwald, H., and H. Green. 1975. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. Cell 6: 331–344.
9. Freeman, A., H. Igel, B. J. Herrman, and K. L. Kleinfeld. 1976. Growth and characterization of human skin epithelial cell cultures. *In Vitro* 12: 352–362.
10. Riegner, D. A., T. McMichael, J. C. Berno, and G. E. Milo. 1976. Processing of human tissue to establish primary cultures in vitro. TCA Manual 2: 273–276.
11. Hayflick, L. 1973. Subculturing human diploid fibroblast cultures. In: P. Kruse and M. K. Patterson (Eds.), *Tissue Culture Methods and Applications*. Academic Press, New York, pp. 220–223.
12. Parshley, M. S., and H. S. Simms. 1950. Cultivation of adult skin epithelial cells (chicken and human) *in vitro*. Am. J. Anat. 86: 163–189.
13. Harnden, D. G. 1960. A human skin culture technique used for cytological examinations. Br. J. Exp. Pathol. 41: 31–37.
14. Karasek, M. A. 1966. *In vitro* culture of human skin epithelial cells. J. Invest. Dermatol. 47: 533–540.
15. Friedman-Kien, A. F., S. Morrill, P. H. Prose, and H. Linenhaber. 1966. Culture of adult human skin: *In vitro* growth and keratinization of epidermal cells. Nature 212: 1583–1584.
16. Green, H. 1978. Cell culture for the study of epithelial cells. Natl. Cancer Inst. Monograph. 48: 259–262.
17. Kitano, Y., and H. Endo. 1977. Differentiation of human keratinocytes in cell culture. In: M. Seiji and I. A. Bernstein (Eds.). *Biochemistry of Cutaneous Epidermal Differentiation*. University Park Press, Baltimore, Maryland, pp. 319–333.
18. Milo, G. E., W. Malarkey, J. Powell, J. Blakeslee, and D. Yohn. 1976. The effects of steroid hormones in fetal calf serum on plating and cloning of human cells in vitro. In Vitro 12: 23–30.
19. Karasek, M. A., and S.-C. Liu. 1977. Keratinization of epidermal cells in cell culture. In: M. Seiji and I. A. Bernstein (Eds.). *Biochemistry of Cutaneous Epidermal Differentiation*. University Park Press, Baltimore, Maryland, pp. 336–353.
20. Bauer, F. W., and R. M. de Grood. 1973. Alteration of subcultured keratinocytes to a keratinizing epithelium. Br. J. Dermatol. 89: 29–32.
21. Marcelo, K. Y. G., J. L. Kaine, and J. J. Voorhees. 1978. Stratification, specialization, and proliferation of primary keratinocyte cultures. J. Cell Biol. 79: 356–370.

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ANALYSIS OF INTRACELLULAR DISTRIBUTION AND BINDING OF BENZO[*a*]PYRENE IN HUMAN DIPLOID FIBROBLASTS*

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SUMMARY

Previous work with low passage synchronized human foreskin fibroblast cell populations has indicated that benzo[*a*]pyrene (BP) can induce a carcinogenic event [3]. BP additionally has shown to damage DNA in logarithmically growing low passage cultures [9]. High passage cells, on the other hand, seem to be refractory to transformation by BP, even though this agent can induce DNA damage, similar to that seen in low passage cells. When low passage cells were treated with BP, the initial binding of the hydrocarbon was primarily to a cytoplasmic protein complex of molecular weight 12,500, while in high passage cells, a major portion of BP was bound to a protein complex of molecular weight 70,000. High-pressure liquid chromatography (HPLC) profiles of the ethyl acetate extractable fractions from the BP-cytoplasmic protein complexes from low and high passage cells demonstrated that the majority of the BP remained unmetabolized. When nuclei were isolated from low and high passage cells prior to the HPLC analysis, the major component (90%) was again unmetabolized BP. The results suggest selective attachment of BP to different cytoplasmic protein

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Abbreviations: PNH, polynuclear hydrocarbon; BP, benzo[*a*]pyrene; HNF, human neonatal foreskin; HPLC, high pressure liquid chromatography; Buffer A, 0.02 M Na phosphate — 0.03 M Tris—HCl — 2.5 mM Na_2 EDTA — 0.5 mM DTT (dithiothreitol), pH 7.5; Buffer B, 0.01 M Tris maleate — 1 mM DTT — 3 mM Ca (Ac), — 2 mM Mg $(Ac)_2$, pH 7.5; BHT, butylated hydroxytoluene; BP-9, 10-diol, 9,10 dihydro-9,10-dihydroxy BP; BP-4,5-diol, 4,5-dihydro-4,5-dihydroxy BP; BP-7,8-diol, 7,8-dihydro-7,8-dihydroxy BP; BP-11, 12-diol, 11,12-dihydro-11,12-dihydroxy BP.

complexes of logarithmically growing human diploid fibroblast cells dependent on the passage level of the cells.

INTRODUCTION

Polynuclear hydrocarbons (PNH) are a class of molecules which must be activated to reactive metabolites in order to function as mutagens or carcinogens. This activation involves the conversion of polynuclear hydrocarbons (PNH)[4], such as BP, to dihydrodiols, epoxides, phenols, quinones and water soluble conjugates [4–6,12,13]. The 7,8-dihydrodiol-9,10-epoxide of BP has been shown to be the major reactive metabolite bound to DNA [14]; BP 7,8-dihydrodiol-9,10 epoxide (*anti*) deoxyguanosine is the major DNA adduct formed in human and bovine bronchial explant tissue [7]. The formation and cellular processing of this covalent DNA adduct is suspected to be a crucial event in BP-induced carcinogenesis.

Earlier reports from our laboratory have shown that BP absorbed into human neonatal foreskin (HNF) cells in culture and first accumulates in the cytoplasm [3]. Labelling of the cells with L-[4,5-^{3}H]leucine before treatment with [7,10-^{14}C]BP indicated that the PNH is initially bound to a cytoplasmic protein complex (unpublished data). Distribution of the BP into the nucleus of treated cells occurs 12 h later [3]. Moreover, BP treatment of HNF cells neoplastically transforms them at passage level 5 (low passage cells), but does not transform cells above passage level 10 (high passage cells) [8]. Optimum transformation is observed when low passage cells are treated with BP 12–24 h prior to entering the S phase; this treatment causes 3.0 breaks/10^8 daltons of DNA [9]. The time required for optimum BP-induced DNA damage coincides with the specific time period in which optimum BP enhancement of focus formation occurs in SV-40 infected transformable cells, i.e., treatment of the cells with BP 12–24 h prior to infection has been shown to enhance focus formation 2-fold [11]. Since SV-40 (viral) DNA does not need to replicate in order to be integrated into the host cell DNA, these observations support the concept that parent (unmetabolized) BP is directly involved in the transformation process within the nucleus. This report presents studies on the nature of association of BP with the cytoplasmic protein complex and nuclei from low and high passage cells, 12–24 h following the initiation of treatment.

MATERIALS AND METHODS

Preparation and isolation of BP-cytoplasmic protein complex

All extraction procedures were carried out under argon and red light to reduce photoxidation and autoxidation.

Passage 5–25 HNF cells were grown, serially subpassaged, and treated with BP as described earlier [3]. After seeding 48–72 h (40–60% cell confluency), the growth medium was replaced with a carcinogen-supple-

mented medium containing 0.125—26 μM [G-^{3}H]BP (16—40 Ci/mmol). After 12 h of treatment, the cells were harvested and washed. The 650 ×*g* cell pellet [3] was homogenized in 2 ml of Buffer A and centrifuged at 100,000 ×*g* for 1 h. The cytoplasmic fraction was partitioned with dextran-coated charcoal in Buffer A, and 2 ml (0.2--0.3 mg/ml protein) of the [G-^{3}H]BP protein complex was applied to a Sephadex G-200 column (58 × 0.9 cm). Elution of 0.5 ml fractions was carried out with 50 ml of Buffer A at a flow rate of 15 ml/h. An aliquot of each fraction was removed and the radioactivity assayed in a Packard Tri-Carb liquid scintillation counter at a tritium counting efficiency of 38%.

HPLC of [G-^{3}H]BP metabolites

Preconfluent cells were exposed to 0.072 μM [G-^{3}H]BP (1 mCi/ml, 27 Ci/mmol) for 12 h and a 100,000 ×*g* fraction was prepared as described above. For the nuclear metabolite profiles, cells were treated with 26 μM [G-^{3}H]BP (1 mCi/ml, 27 Ci/mmol) for 24 h, and nuclei were prepared by a modification of the procedure of Chaveau et al. [2]. The nuclear pellet was suspended in Buffer B and contrast-interference Nomarski microscopic examination of the nuclear suspension indicated a 35% recovery of nuclei.

Extraction of either the BP-cytoplasmic protein complex or the nuclear fraction was completed with 3 vols. of ethyl acetate in the presence of 0.8 mg/ml BHT; the organic phase was passed over anhydrous sodium sulfate, filtered, dried under argon and dissolved in 0.5 ml of acetone/methanol (2 : 1 v/v). Aliquots were removed for counting and the remaining sample was dried under argon and stored at −90°C. The sample was reconstituted with methanol/acetone/DMSO (2 : 1 : 1 by vol.), non-radioactive BP metabolite standards added, and the extract chromatographed on a Dupont Instruments Model 848 High Pressure Liquid Chromatograph with 4 mm × 30 cm μ-Bondapak C_{18} column (Waters Associates) using an isocratic elution solvent of methanol/water/ethyl ether (66.3 : 30.4 : 3.3, by vol.) at a flow rate of 1.4 ml/min. The effluent was monitored by UV spectrometry to identify metabolites, which were quantitated by collecting appropriate fractions of the effluent for liquid scintillation analysis. Six second fractions were collected for 11—12 min, then 12-s fractions were collected for 8—9 min and lastly, 60-s fractions were collected until the completion of the chromatographic run. Typical retention times in minutes for each metabolite were: BP-9,10-diol, 3.6; BP-4,5-diol, 5.5; BP-11,12-diol, 5.8; BP-7,8-diol, 6.5; BP-1,6-quinone, 8.6; BP-11,12-quinone, 9.1; BP-3,6-quinone, 9.6; BP-6,12-quinone, 11.0; BP-9-phenol, 16.5; BP-3-phenol, 19.1; BP, 37.5. The overall recovery of radioactivity from the column was greater than 90%.

RESULTS

[G-^{3}H]BP-protein complexes isolated from the cytoplasm of low and high passage cells were chromatographed on a Sephadex G-200 column.

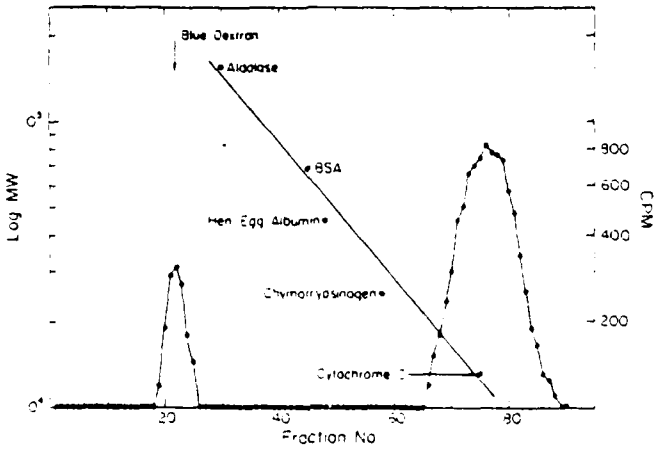

Fig. 1. Sephadex G-200 chromatography of a [G-^{3}H]BP-cytoplasmic protein complex from low passage cells. The cytoplasmic fraction was prepared from passage 5 HNF cells treated with [G-^{3}H]BP for 12 h. 50,000 dpm were applied to a Sephadex G-200 column (58 × 0.9 cm) and eluted with 0.01 M Na phosphate — 0.3 M Tris—HCl — 2.5 mM Na_2EDTA — 5 mM DTT (pH 7.5). 0.5-ml fractions were collected and the radioactivity was assayed. Blue Dextran — 200,000; Aldolase — 158,000; Bovine Serum Albumin — 67,000; Hen Egg Albumin — 45,000; Chymotrypsinogen — 25,000; and Cytochrome c — 12,500 served as molecular weight standards.

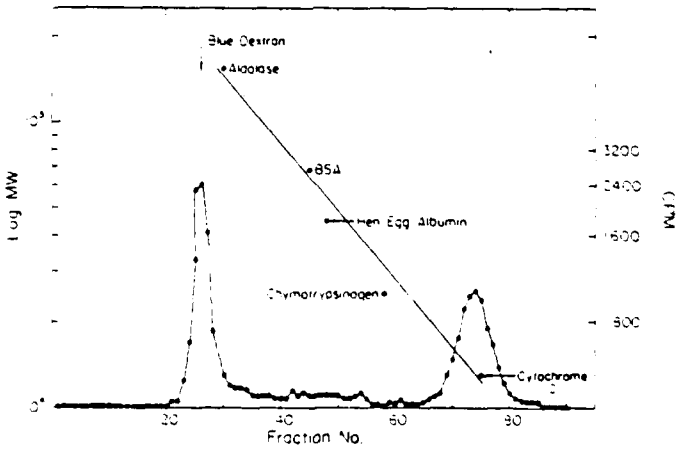

Fig. 2. Sephadex G-200 chromatography of a [G-^{3}H]BP-cytoplasmic protein complex from high passage cells. The cytoplasmic fraction was prepared from passage 25 HNF cells treated with [G-^{3}H]BP for 12 h. 100,000 dpm were applied to a Sephadex G-200 column (58 × 0.9 cm) and 0.5 ml fractions were collected as described under Fig. 1.

In low passage cells, a major portion of BP was associated with a protein complex of molecular weight 12,500. The ratio of area under the low molecular weight peak to the high molecular weight peak was 6.5 (Fig. 1). In high passage cells, a major portion of BP was associated with a protein complex of molecular weight 200,000 and the ratio of the area under the

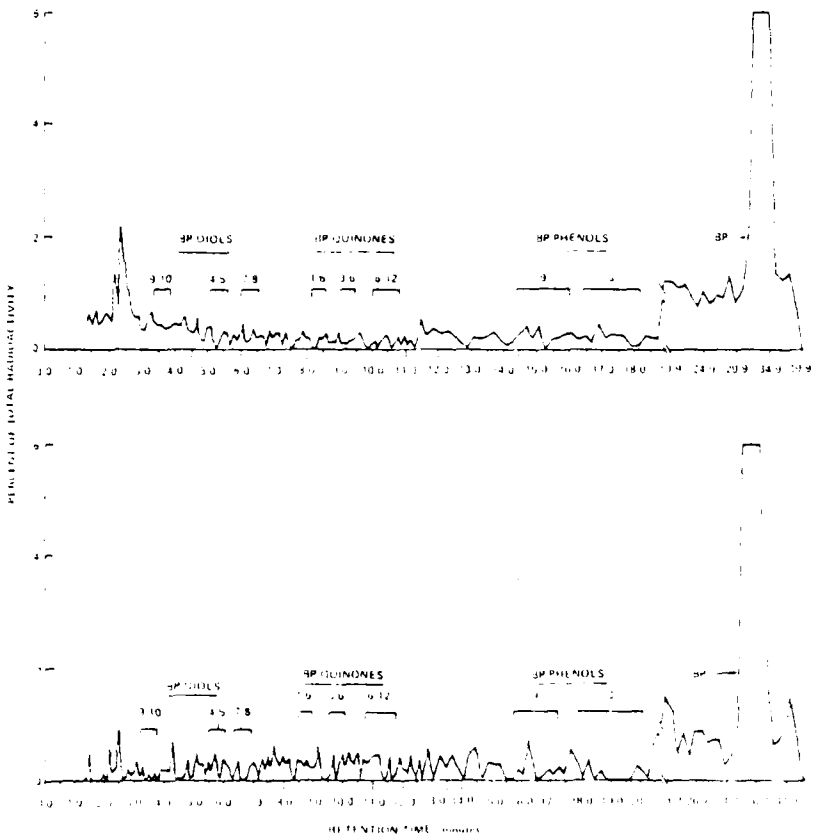

Fig. 3. HPLC profiles of the ethyl acetate extractable radioactivity from the total cytoplasmic protein-hydrocarbon complexes of low and high passage cells. Non-radioactive BP metabolite standards were cochromatographed with the radioactive extract for metabolite identification. An isocratic elution solvent of methanol/water/ethyl ether (66.3 : 30.4 : 3.3, by vol.) was employed at a flow rate of 1.4 ml/min and fractions collected for liquid scintillation spectrometry. (See Material and Methods for details.) Upper panel: Profile of the organic extract of [G-^{3}H]BP-total cytoplasmic protein complex from high passage human diploid fibroblasts. A total of 6300 dpm were applied to the column. Lower panel: profile of the organic extract of [G-^{3}H]BP-cytoplasmic protein complex isolated from low passage human diploid fibroblasts. A total of 4000 dpm were applied to the column. Note the discontinuity in the retention time scale.

high molecular weight peak to the low molecular weight peak was 1.8 (Fig. 2).

The total cytoplasmic protein complex isolated from low passage human fibroblast cells exposed to [G-^{3}H]BP was extracted with ethyl acetate, and the non-covalently bound hydrocarbon and its metabolites were co-chromatographed on a reverse phase column by high-pressure liquid chromatography with authentic reference standards. In the metabolite profiles of

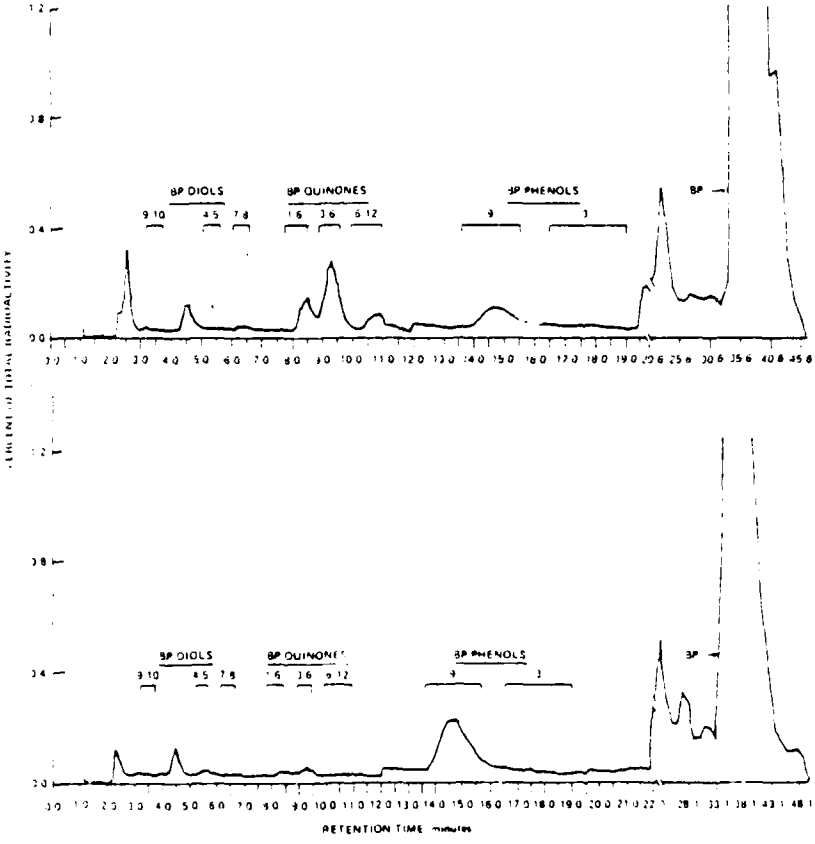

Fig. 4. HPLC profiles of the ethyl acetate extractable radioactivity from nuclei of low and high passage human diploid fibroblasts exposed to [G-^{3}H]BP. Non-radioactive BP metabolite standards were cochromatographed with the radioactive extract for metabolite identification. (See Fig. 3, Materials and Methods for details). Upper panel: profile of the organic extract from nuclei of high passage cells. A total of 1.263×10^6 dpm were applied to the column. Lower panel: Profile of the organic extract from nuclei of low passage cells. A total of 580,850 dpm were applied to the column. BP-11,12-diol and BP-11,12-quinone cochromatographed with BP-4,5-diol and BP-3,6-quinone, respectively.

BP-treated low passage cells (Fig. 3), unmetabolized BP was the only radiolabeled fraction. Similar results were obtained with BP radioactivity isolated from the total cytoplasmic protein complex of high passage cells, except that a small peak of radioactivity eluted prior to the BP-9, 10-diol (Fig. 3).

The radioactivity associated with isolated nuclei of low and high passage cells treated with [G-^{3}H]BP was chromatographed as described above (Fig. 4). The nuclei from high passage cells had radioactivity which co-chromatographed with BP-1, 6; 3,6 and 6,12-quinones and BP-9-phenol; 89% of the counts were associated with BP; however, the radioactivity isolated from [G-^{3}H]BP-treated low passage cells co-chromatographed with BP-9-phenol, with 90% of the counts eluting with BP. The unknown peaks at 2.4 and 4.5 min represent void volume radioactivity (pre-BP-9,10-diol) and an unidentified metabolite, respectively. Co-chromatography with BP-11,12-diol and BP-11,12-quinone indicated that the 4.5 min peak represented neither of these potential metabolites.

DISCUSSION

When low passage HNF cells are treated with BP, the PNH accumulates in the cytoplasm, before localizing in the nucleus 24 h after exposure. Sephadex G-200 gel chromatographic separation of the cytoplasmic protein complexes from low passage and high passage cells indicated the distribution of BP between protein complexes of molecular weights, 12,500 and 200,000. In low passage cells, the amount of BP associated with the low molecular weight protein complex was 4–7 times that associated with the high molecular weight protein complex. However, in high passage cells, the amount of BP associated with the high molecular weight protein complex was 0.8—1.8 times that associated with the low molecular weight protein complex. HPLC analysis of the BP radioactivity separated from the BP-total cytoplasmic protein complex of low passage and high passage cells indicated that the major fraction was the parent BP. This is in contrast to a previous study [10], in which active metabolites of 3′-methyl-4-dimethyl amino azobenzene were shown to bind to a cytosol protein complex from rat liver. It was also interesting to observe that unmetabolized [G-^{3}H]BP made up the major fraction of the PNH associated with the nuclei from low passage and high passage cells. The minor peaks observed in the HPLC metabolite profiles may be a result of autoxidation of the sample, although all procedures were performed under red light, argon, and in the presence of an antioxidant.

We have observed minor differences in the metabolites non-covalently bound to DNA, in low and high passage cells. Also, as described above, we have observed binding of BP to different cytoplasmic protein complexes in low and high passage cells. The transport of BP into the nucleus of these cells may involve an activation of the BP-cytoplasmic protein complex, similar to that observed with the steroids. Only binding of BP

to the lower molecular weight protein complex (the predominant complex in low passage cells) may result in an activation of the complex. Therefore, the accessability of BP metabolites to specific nuclear binding sites in low and high passage cells may be different and may account for the susceptibility or refractoriness to BP-induced carcinogenesis of human fibroblast cells in vitro.

ACKNOWLEDGEMENT

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REFERENCES

1 Baird, W.M. and Diamond, L. (1978) Metabolism and DNA binding of poly-cyclic aromatic hydrocarbons by human diploid fibroblasts. Int. J. Cancer, 22, 189–195.
2 Chaveau, T., Moule, Y. and Rouiller, C. (1956) Isolation of pure and unaltered liver nuclei. Morphology and biochemical composition. Exp. Cell Res., 11, 315–321.
3 Ekelman, K.B. and Milo, G.E. (1978) Cellular uptake, transport and macromolecular binding of benzo(a)pyrene and 7,12-dimethyl benz(a)anthracene by human cells *in vitro*. Cancer Res., 38, 3026–3032.
4 Fahl, W.E., Jefcoate, C.R. and Kasper, C.B. (1978) Characteristics of benzo(a)pyrene metabolism and cytochrome *P*-450 heterogeneity in rat liver nuclear envelope and comparison to microsomal membrane. J. Biol. Chem., 253, 3106–3133.
5 Fox, C.H., Selkirk, J.K., Price, F.M., Croy, R.G., Sanford, K.K. and Cottler Fox, M. (1975) Metabolism of benzo(a)pyrene by human epithelial cells *in vitro*. Cancer Res., 35, 3551–3557.
6 Huberman, E. and Sachs, L. (1973) Metabolism of the carcinogenic hydrocarbon benzo(a)pyrene in human fibroblast and epithelial cells. Int. J. Cancer, 11, 412–418.
7 Jeffrey, A.M., Weinstein, I.B., Jennette, K.W., Grzeskowiak, K., Nakanishi, K., Harvey, R.G., Autrup, H. and Harris, C. (1977) Structures of benzo(a)pyrene-nucleic acid adducts formed in human and bovine bronchial explants. Nature, 269, 348–350.
8 Milo, G.E. and DiPaolo, J.A. (1978) Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment. Nature, 275, 130–132.
9 Milo, G.E., Blakeslee, J., Yohn, D. and DiPaolo, J.A. (1978) Biochemical activation of aryl hydrocarbon activity, cellular distribution of PNH metabolites and DNA damage by PNH products in human cells *in vitro*. Cancer Res., 38, 1638–1644.
10 Mainigi, K.D. and Sorof, S. (1977) Evidence for a receptor protein of activated carcinogen. Proc. Natl. Acad. Sci. USA, 74, 2293–2296.
11 Milo, G.E., Blakeslee, J., Hart R. and Yohn, D.(1978) Chemical carcinogen alteration of SV-40 virus induced transformation of normal human cell populations *in vitro*. Chem.-Biol. Interact., 22, 185–197.
12 Selkirk, J.K., Croy, R.G., Whitlock, J.P. and Gelboin, H.V. (1975) *In vitro* metabolism of benzo(a)pyrene by human liver microsomes and lymphocytes. Cancer Res., 35, 3651–3655.
13 Yang, S.K., Deutsch, J. and Gelboin, H.V. (1978) Benzo(a)pyrene metabolism: Activation and detoxification. In: Polycyclic Hydrocarbons and Cancer, Vol. 1, pp. 205–231. Editors: H.V. Gelboin and P.O.P. Ts'o. Academic Press, New York.

14 Weinstein, I.B., Jeffrey, A.M., Leffler, S., Pulkrabek, P., Yamasaki, H. and Grunberger, D. (1978) Interactions between polycyclic aromatic hydrocarbons and cellular molecules. In: Polycyclic Hydrocarbons and Cancer, Vol. 2, pp. 3—36. Editors: H.V. Gelboin and P.O.P. Ts'o. Academic Press. New York.

ESTABLISHMENT OF PROLIFERATING HUMAN EPITHELIAL CELLS IN VITRO FROM CELL SUSPENSIONS OF NEONATAL FORESKIN

Submitted by

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I. INTRODUCTION

The following procedure has been successfully applied to many different human tissues. Tissue samples are obtained from cooperating hospitals. Using the collection techniques described here, we can retain excellent viability from 96 hr up to 5 days (postcollection) depending on the tissue of choice and source of tissues. The use of this procedure permits the establishment of epithelial cell cultures from cell suspensions not requiring explant growth or the addition of extrinsic modulating growth factors. Moreover, epithelial cell colonies can be produced at a low density or high density directly from the cell suspension.

Key words: primary; epithelial cells; cell suspensions.

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Procedure No. 4 1 5 1 1

II. MATERIALS

Minimum essential medium (MEM) Eagle with Hanks' salts (HBSS) (GIBCO[1]) and 25 mM HEPES; without glutamine and $NaHCO_3$. To 100 ml of the medium, add 1 ml non-essential amino-acid mixture (10 mM, Micro[2]), 1 ml sodium pyruvate (100 mM solution[3]), 0.1 ml Gentocin (50 mg per ml, Schering[3]), 1 ml L-glutamine (200 mM[4]), and titrate with 8.8% $NaHCO_3$ solution (sterile, carbonate-free) to pH 7.2. Designated complete growth medium (CM).

MEM Eagle without magnesium and calcium[1] (not supplemented unless mentioned in Procedure section), for suspension (spinner) cultures. Designated spinner medium (SM).

Dulbecco's LoCal medium, Biolabs.[4] Supplement exactly as CM. Designated LoCal.

Trypsin, lyophilized, No. TL 13 BP Worthington[5] (1% solution made up in MEM Eagle HBSS medium)

Essential vitamin mixture, 100X[2]

Collagenase (CLS), No. 4197.[5] Suspend 1 g collagenase in 100 ml MEM Eagle HBSS, pH 7.2; dissolve with magnetic stirrer at 4° C; centrifuge 10,000 × g at 12° C for 10 min; and filter through a 0.22-μm filter (No. 7103 Falcon[6]).

Fetal bovine serum (FBS)[7] [evaluated for steroid composition (1), unsaturated fatty acid composition (2), mycoplasmal contamination, etc. (3), and growth properties on indicator cells (4)]

Stirring bars, Teflon-molded, magnetic, ¼-inch long, ⅝-inch diameter, No. 6006 Bellco[8]

Culture plates, four wells per plate, 28-cm², No. 3004[6] or No. FB-4-TC Linbro[9]

Scalpels, disposable, sterile, No. 32 390-0222 AHS[10]

Tissue culture flasks: 75-cm², No. 3024[6] or No. 5375[6]; or 25-cm², No. 25100 Corning[11]

Plastic pipettes, measuring (Mohr), plugged: 5-ml, No. 7532; 10-ml No. 7548[6]

Centrifuge tubes, conical, plastic, 15-ml, No. 3013-000[11]

Ethylenediamine tetracetate (EDTA), tetrasodium salt[12]

[1] Grand Island Biological Co., Grand Island NY.
[2] Microbiological Associates, Walkersville MD.
[3] Schering Veterinary Surgical Corp., Kenilworth NJ.
[4] BioLabs, Inc., Northbrook IL.
[5] Worthington Biochemical Corp., Freehold NJ.
[6] Falcon Products, Oxnard CA.
[7] No recommendation offered; each user will have to determine which supplier can provide ample volume of useful sera.
[8] Bellco Glass, Inc., Vineland NJ.
[9] Linbro Scientific, Hamden CT.
[10] American Hospital Supply, Obetz OH.
[11] Corning Glass Works, Corning NY.
[12] Sigma Chemical Co., St. Louis MO.

III. PROCEDURE

A. Collection of human tissue

1. Supply the operatir g room with several 4-oz bottles containing 10 ml CM at pH 7.2 supplemented with 5% FBS and 0.1 ml Gentocin per 100 ml medium. These bottles may be stored at 12° C for many

weeks (in our hands, 3 to 4 weeks, depending on composition of the glass). If there is a drastic change in pH, discard the bottles, i.e. if the pH rises above 7.2 (color shifts from red to purple) or falls below 6.8 (color shifts from red to yellow).

2. Collect tissue on the average of 3 or 4 times a week. We have found that when the tissue is kept in CM plus 5% FBS at 12° C, 95% viability is retained up to 48 hr after collection.

B. Processing of tissue

1. Charge each of four wells of a 28-cm² culture plate with 5 ml CM.
2. Place the tissue from the collection bottles into the first well, swirling the medium to wash the tissue.
3. Transfer the tissue to the second well and wash.
4. In the third well, cut the tissue into three or four segments and wash.
5. Transfer segments to the fourth well and mince with two scalpels into 2-mm pieces.
   a. Swirl the medium to rinse the tissue.
   b. Suck off the medium with a narrow-mouth pipette leaving only the minced tissue.
6. Defrost a 5-ml vial of 1% collagenase and add to the well containing the minced pieces. Have ready a 75-cm² flask containing 15 ml CM supplemented with 20% FBS. Transfer the minced tissue and collagenase to the preincubated flask, thereby diluting collagenase to 0.25%.
7. Incubate the tissue at 37° C in a 4% CO_2 environment overnight (16 hr). For a period of 5 to 7 hr of incubation, use 0.5% collagenase.
8. Transfer the digest into a 15-ml plastic or glass conical centrifuge tube. (Use plastic pipette with a 1.5-mm diameter aperture.)
9. Centrifuge the sample for 7 min at 650 × *g* at 4° to 12° C.
10. Resuspend the pellet in 5 to 10 ml CM supplemented with 20% FBS; recentrifuge again as described in step 9.
11. Repeat step 10.
12. Preincubate a 75-cm² flask containing 10 ml CM supplemented with 20% FBS for 30 to 45 min at 37° C in a 4% CO_2 environment.
13. Suspend the pellet obtained from step 11 in 5 ml CM at 20% FBS. Seed one 75-cm² flask or three or four 25-cm² flasks with the cell suspension.
14. Incubate the flasks at 37° C in a 4% CO_2 environment.
15. Two days later, rinse the primary culture with CM and refeed with CM supplemented with 20% FBS.
16. Three to five days postseeding, check for epithelial colonies.
17. Fibroblasts also will be present in these cultures. Three to five days after seeding, when epithelial colonies are well established, selectively trypsinize the cultures to remove the fibroblasts. PRECAUTIONARY NOTE: This step is a critical procedure and particular attention must be paid to it. The epithelial islands should be left relatively undisturbed.
18. Decant growth medium from the mix cultures and rinse with 10 ml CM.
19. At this time, remove from the freezer the trypsin prepared as a 1% solution in CM at pH 7.2.
    a. Dilute with CM to 0.1%.
    b. Add 1 ml 0.1% trypsin to the cell sheet.
    c. Incubate at 21° C (room temperature) for approximately 30 sec.
    d. Observe the cell sheet under 10X magnification to determine when fibroblasts lift off the substratum. The epithelial patches will remain attached to the flask.
20. To stop the action of trypsin, add 10 ml CM supplemented with 20% FBS and use this medium to wash the cell sheet to remove fibroblasts.
21. Decant medium and repeat step 20.
22. Do not attempt to remove all fibroblasts during this first trypsinization. When the edges of the epithelial patches begin to retract, immediately stop trypsin action. It is better to repeat steps 18–20 the next day than to continue to remove all the fibroblasts at this time.
23. Refeed the flasks containing mainly epithelial colonies with CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium. The lowered FBS supplementation retards the growth of any remaining fibroblasts while the vitamin supplementation encourages epithelial growth.
24. Repeat trypsinization procedures two to four times at approximately 3-day inter-

vals or as necessary to free cultures from fibroblasts.

C. Subpassaging epithelial cells

1. Place epithelial cells for 2 to 3 days on LoCal containing additives and supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml of medium. Pretreatment with calcium-deficient medium greatly facilitates the lifting off of the epithelial cells from the substratum during trypsinization.
2. Decant the LoCal medium, rinse the cell sheet with 10 ml SM containing 0.02% EDTA, and incubate at 37° C for 3 to 5 min. During this time epithelial cells will begin to separate along their boundaries but will continue to remain attached to the substratum. An increase in refractoriness along the outer limits of each cell will be noticed.
3. Decant the SM and add 1 ml 0.1% trypsin made up with SM containing 0.02% EDTA.
4. After a few seconds, stop the trypsinization by decanting the SM-EDTA medium and add CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium. Transfer the cell suspension to a 15-ml plastic conical centrifuge tube.
5. Centrifuge at 650 × *g* for 7 min; decant supernate.
6. Resuspend the pellet with CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium, and seed equally into four 25-cm² flasks, or one 75-cm² flask. We use 5 ml of medium containing the cell suspension. Cells should be seeded at high density.
7. After 10 min, gently swirl dishes or flasks to encourage cells to adhere to each other.
8. Return the cultures to the incubator and do not disturb for 3 to 4 days.
9. After 3 to 4 days, examine the cultures for growth of epithelial colonies.

IV. DISCUSSION

There are several critical steps in the procedure that will mean the difference between success and failure. During the initial phase of collection of the tissue, make sure that the pathologist or surgeon does not place the samples into phosphate buffered saline (PBS) or physiological saline, such as Kreb's solution. The carrier medium defined here will keep the tissue viable up to 4 or 5 days at a 95% efficiency. *Do not use penicillin-streptomycin, nystatin, mycostatin, amphotericin B, tetracyclines, etc.; they will kill the cells.*

All plastic ware, i.e. petri dishes and 75-cm² flasks, should be kept in a constant environmental room at 72° C at a relative humidity (R.H.) of 75%. The flasks under these conditions will remain in acceptable condition for 2½ years. The single well and multiwell dishes can be used for up to 6 months when stored under these conditions. All pipettes used to pipette cells should have an aperture of 1.5-mm diameter. The FBS must be evaluated before use (1). Collagenase and purified trypsin prepared specifically for tissue culture applications should be screened for mycoplasma (2). Photographs of vertical stratification of epithelial patches and tables of growth kinetics are presented in Milo, Ackerman and Noyes (4).

V. REFERENCES

1. Milo, G., W. Malarkey, J. Powell, J. Blakeslee, and D. Yohn. 1976. Effects of steroid hormone and fetal bovine serum on plating and cloning of human cells in vitro. In Vitro 12: 23–30.
2. Huttner, J., G. E. Milo, R. V. Panganamala, and D. G. Cornwell. 1978. Fatty acids and the selective alteration of in vitro proliferation in human fibroblast and guinea pig smooth muscle cells. In Vitro 14: 854–859.
3. Barile, M. F., and J. Kern. 1971. Isolation of mycoplasma arginini from commercial bovine sera and its implication in contaminated cell cultures. Proc. Soc. Exp. Biol. Med. 138: 432–437.
4. Milo, G. E., G. A. Ackerman, and I. Noyes. Growth and ultrastructural characterization of proliferating human keratinocytes in vitro without added extrinsic factors. In Vitro 16: 20–30.

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CONSTRUCTION AND PARTIAL CHARACTERIZATION OF LATE T7 MUTANT PROMOTERS. K. Chapman*, (SPON: J. Adler) Dept. of Biochem. University of Wisconsin, Madison, WI 53706.

The sequence 5' TAATACGACTCACTATAGPuPuPuNA is common to all T7 late promoters. To study the interaction of T7 RNA polymerase with this sequence, several mutant promoters have been constructed by cloning segments of the promoter, cleaved at the Hinf I site (GACTC) which is present in all late T7 promoters, into various plasmid backgrounds. For starting material, the 116 bp Hpa II-Taq I fragment which spans the promoter at 14.8% of T7 was cloned into the Eco RI site of pVH51. Eco RI sites were regenerated at the ends of this fragment by filling in the sticky ends before ligation. Multiple copies (up to hexamer) of this Eco RI fragment were then cloned. The isolated fragment was cut with Hinf I to yield a 106 bp fragment which contained the right-hand 16 bp of the promoter. This 106 bp fragment was then cloned into several sites in pBR322. Careful choice of the cloning sites enables the construction of mutant promoters which contain some, but not all, of the natural left-hand promoter bps. Some of these clones are active in an in vitro T7 transcription system. Restriction mapping and preliminary sequencing show that it is not necessary for the Hinf I site to be intact for the sequence to be an active promoter. Moreover, neither the first 10 nor the last 13 bp alone are sufficient for activity. (Supported by NIH and NSF grants to R.D. Wells.)

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HETEROGENEITY OF THE 5'-TERMINUS OF CONALBUMIN MESSENGER RNA. Beth Genye Cohen* and William H. Eschenfeldt* (SPON: Shung Kai Chan). Univ. of Ky. College of Medicine, Lexington, KY 40536

Previously this laboratory determined that the 5'-terminus of hen oviduct ovalbumin mRNA was heterogeneous, apparently resulting from different sites of initiation of transcription. In order to determine whether such heterogeneity was a common phenomenon in hen oviduct mRNAs, we used a similar method in the examination of conalbumin mRNA, the next most abundant mRNA in the hen oviduct. The method involves purification of conalbumin mRNA by hybridization to the complementary DNA contained in the conalbumin plasmid, pCon-1, attached to cellulose, isolation of 5'-terminal, capped T_1 oligonucleotides by selective binding to anti-m^7G-Sepharose, labeling with $[^{32}P]$pCp and RNA ligase, separation by two-dimensional gel electrophoresis, and sequencing by conventional RNA sequencing procedures. This method has the advantage of eliminating ambiguities of the primed-synthesis-dideoxy method. On the two dimensional gel, a number of T_1 oligonucleotides were observed. The major oligonucleotide had the sequence m^7GpppNpUpCpApCpApG, which would result from transcription from the putative initiation site on the genome. A second oligonucleotide had the sequence m^7GpppNpNpApG. This is consistent with transcription beginning at the next purine three bases downstream from the putative initiation site. (Supported by Grant # GM20818 from the NIH).

CARCINOGENS AND ANTI-TUMOR SUBSTANCES (213–216)

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THE INFLUENCE OF AGE ON THE ACTIVATION AND METABOLISM OF AFLATOXIN B_1 BY LIVER FROM MALE AND FEMALE RATS. Andrew Jayaraj* and Tom Diller*. (SPON: W.A. Riddle). Illinois State University, Normal, IL 61761

Aflatoxin B_1 (AFB_1), a chemical pro-carcinogen, is metabolised by the P450 mono-oxygenase system in liver to its ultimate carcinogenic form. The effect of aging on the metabolism of AFB_1 by the liver from 5- to 30- month old male and female Fischer 344 rats was studied. The Ames Salmonella/microsome test was used to quantify the conversion of AFB_1 to mutagenic compounds by liver homogenates from rats of various ages. The number of revertants produced decreased 40 to 60% between 12 months and 27 months of age. No age related change in the conversion of AFB_1 to mutagenic compounds by liver homogenates from female rats was observed. The formation of AFB_1-DNA adducts by liver homogenates from male rats also decreased with increasing age; however, no change on the formation of AFB_1-DNA adducts was observed with liver homogenates from female rats. The metabolism of AFB_1 by liver homogenates from male rats to chloroform-soluble, water-soluble , and ethanol insoluble metabolites also decreased as a function of age, while the metabolism of AFB_1 by female liver did not change. The results of these experiments indicate that the metabolism and activation of AFB_1 by liver from male rats decreased as a function of age. This decrease appears to arise from changes in the cytochrome P-450 levels in liver. (Supported in part by NIH Grant CA 24856).

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BINDING OF BENZO(A)PYRENE TO A CYTOPLASMIC LIPOPROTEIN COMPLEX IN THE HUMAN SKIN FIBROBLAST CELLS. Raman Tejwani* and George E. Milo. The Ohio State University, Columbus, Ohio 43210.

Previous work with low passage (LP) human neonatal foreskin (HNF) fibroblast cells has indicated that benzo(a)pyrene (BP) can induce a carcinogenic event. When the HNF cells were treated with BP, the carcinogen was initially bound to a cytoplasmic protein complex prior to transport into the nucleus. In the LP cells, BP was non-covalently associated with a cytoplasmic protein complex of molecular weight 12,500 as determined by Sephadex G-200 column chromatography. When the complex isolated from LP cells was centrifuged on a potassium bromide gradient (ρ, 1.006-1.279) at 40,000xg for 24 hrs, the radiolabel was distributed between two major lipoprotein fractions. In excess of 30% of the recovered radiolabel was associated with the low density lipoprotein fraction (ρ, 1.073) and about 25% of the radiolabel was associated with the high density lipoprotein fraction (ρ, 1.125). Since there was no significant reduction in the uptake of BP by cells grown in lipoprotein deficient serum, the lipoprotein complex to which BP was bound was not derived from the fetal bovine serum in which the cells were grown. Thus, in the transformable LP cells, BP is associated with a lipoprotein complex that functions to transport the carcinogen into the nucleus of these cells. (Supported in part by Air Force Office of Scientific Research F 49620-80-C-0085 and EPA R-8006638-01).

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DIFFERENCES IN THE BINDING OF 3-METHYLCHOLANTHRENE AND PHENOBARBITAL TO RAT LIVER CYTOSOLIC AND NUCLEAR PROTEIN FRACTIONS. Brian Tierney* and Edward Bresnick. University of Vermont College of Medicine, Dept. Biochemistry and Vermont Regional Cancer Center, Burlington, VT 05405.

The inducers of cytochrome P-450b and P-450c, phenobarbital (PB) and 3-methylcholanthrene (3-MC) respectively, have been studied in their interaction with subcellular fractions from rat liver. Binding of 3-MC to rat liver cytosol was observed using DEAE A-50 sephadex chromatography, charcoal dextran analysis, sucrose density gradients and DNA-cellulose chromatography, while no interaction of PB to rat liver cytosol was observed under comparable conditions. The binding of 3-MC to rat liver nuclear fractions has also been studied using DNA-cellulose chromatography; higher levels of binding were found in the nuclear fractions (250-500 fmoles 3-MC per mg protein) compared with cytosolic proteins (20-30 fmoles 3-MC per mg protein). The uptake and intranuclear distribution of 3-MC and PB were markedly different after incubation with whole nuclei; 30% of the available 3-MC but only 3% of the available PB radioactivity became associated with nuclei. Of this radioactivity, approximately 50% of the 3-MC radioactivity was associated with the salt resistant nuclear pellet and [illegible] of the PB was associated with the soluble nuclear fraction. The data suggest that it is unlikely that a cytosolic receptor-mediated process requiring high affinity binding is involved in phenobarbital induction of cytochrome P-450b. Supported by NIH grant CA 20711.

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INHIBITORS OF BENZO(A)PYRENE MUTAGENICITY TOWARDS SALMONELLA TYPHIMURIUM. Paul O. Sullivan* and Luz M. Calle* (SPON: L. Packer) Ohio University, Athens, OH 45701.

Antioxidants and several other compounds, some of which have been found to inhibit carcinogenicity, were screened for their effectiveness as inhibitors of benzo(a)pyrene (BP) mutagenicity towards Salmonella typhimurium TA98 in the Ames test. A total of 32 compounds were tested. In the assay, metabolic activation of BP (8.2 nmoles/plate) was mediated by the S9 fraction from β-napthoflavone induced rat livers. Among compounds which are known to inhibit carcinogenicity, retinol, phenothiazine, disulfiram, phenethylisothiocyanate and phenylisothiocyanate were the most effective inhibitors of BP mutagenicity, acting at equimolar concentrations. Several other compounds showed inhibition at higher concentrations of antioxidant and the remainder showed little or no inhibition. Dose response curves have been obtained for the 18 most active compounds. No general pattern of inhibition is obvious from our studies, inhibitors are not drawn from any single class of compounds, nor does a particular compound necessarily appear to inhibit more than one mutagen. Additional studies have been carried out on the inhibition of the direct acting mutagens 2-nitrofluorene and benzo(a)pyrene-4,5-oxide. These studies indicate the possible nature of the mechanism of inhibition. (Supported by NIH Grant CA-22209)

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INDUCTION OF NEOPLASIA AFTER IN VITRO EXPOSURE OF HUMAN EPITHELIAL CELLS TO CARCINOGENS. George Milo, Inge Noyes, John Donahoe, and Steven Weisbrode. Depts. of Physiological Chemistry, Veterinary Pathobiology, and Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210.

These studies demonstrate that known carcinogens can alter normal foreskin epithelial cells to the extent that neoplasia can be evaluated on chick embryonic skin, (CES), in vitro. Normal foreskin epithelial cells seeded on CES did not invade the CES. Human nasopharyngeal and chorio-carinoma cells when seeded on CES invaded the tissue. Normal epithelial cells grown in Eagles's modified MEM supplemented with 20% FBS, (CM), were then transferred to MEM minus arginine-glutamine medium supplemented with 1.0 U/ml of insulin and 10% dialyzed FBS, (DM), for 2 hrs. Within this time period 56% of the cells were radiolabeled with ^{3}H-thymidine, (S). In a randomly proliferating population grown on CM over a 24 hr period <1% of the cells in S, were radiolabeled. Upon transfer of the cells from DM to CM either β-propiolactone or propane sultone, (7.5 ug/ml), MNNG, (0.4 ug/ml) or aflatoxin B_1 (2.5 ug/ml), were added to the cell populations, or the cells were exposed to UV 254nm at 5.0 J/m^2 at a fluence of 1.2 J/m^2/sec. After 8 hrs of chemical carcinogen treatment or at the completion of exposure of the cell population to UV, the experimental medium was removed and MEM+20% FBS added to the cells. Two-3 weeks later the cultures were passaged twice. These populations were seeded onto CES, or into LoCal supplemented 0.33% agar. Transformed cells passaged in culture exhibited colony forming frequency in soft agar for, AffB_1 was 41, MNNG was 32, PrS was 58, βPl was 23 and for UV was 100 per 10^5 cells. Tumors that invaded CES were interpreted as simulated squamous cell carcinomas. Induction of carcinogenesis in human epithelial cells is similar to the induction process in fibroblasts, however, the program leading up to the induction process and time in culture for fixation and expression of neoplasia, is different. (This work was supported in part by NCI-NIH R01-CA-2507).

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A SUITABLE RAPID IN VITRO ORGAN CULTURE SYSTEM TO EVALUATE NEOPLASIA EXHIBITED BY CARCINOGEN TRANSFORMED HUMAN FIBROBLASTS. John Donahoe, Inge Noyes, Steven Weisbrode, Dorothy Schumm and George Milo. Depts of Physiological Chemistry, Veterinary Pathobiology and Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210.

Recent experiences with nude mice have taught us that the mice exhibit wide variations in response to injection of transformed human cells. Each mouse responds in a different manner dependent upon the genetic background of the mouse. Moreover, the length of time necessary to evaluate the neoplastic potential of the transformed cells is 5-6 weeks. The average lifespan of the nude mouse under barrier conditions is 1-1 1/2 yr creates logistic problems in feed and housing costs. We have expanded upon a procedure for evaluating the neoplastic potential of the chemical carcinogen transformed cells on Chick-embryonic skin, (CES), in vitro (Noguchi, et al., Science 199:980-983, 1978), as a suitable substitute, for the nude mouse assay system, (Gianovella, et. al., 24:103-113,1979). Several chemical carcinogens, aflatoxin B_1, propane sultone, β-propiolactone, benzo(a)pyrene, MNNG, or methyl azoxy methanol acetate were used to induce carcinogenesis in human fibroblasts, (Milo and DiPaolo Nature, 275, 130-132, 1978). Treatment of the cells in S with the carcinogens followed by serially passaging the cells in culture for 16-20 PDL produced cell populations that grow in soft agar. These colonies were isolated and seeded onto CES; three days later the CES were fixed in Bouins, stained and evaluated. There was a 100% correlation between tumor formation in the nude mouse and invasion of the CES. Histopathological examination of the microtumors revealed that they were simulated fibrosarcomas. The cells from the untreated controls did not invade the CES. (This work was supported in part by AFSOR F 49620-80-C0085).

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A Comparison of Expression of Neoplastic Potential of Carcinogen-Transformed Human Fibroblasts In Nude Mice and in Chick Embryonic Skin

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Key Words: Chemical Carcinogens, neoplasia, human cell transformation

SUMMARY

Human foreskin fibroblasts transformed by representative chemicals from 5 different classes of chemical carcinogens some requiring enzymatic activation and direct acting carcinogens, produced cell populations that exhibited anchorage-independent growth and expression of neoplastic potential in either nude mice or chick-embryonic skin, (CES). There is a high degree of correlation between tumor incidence and invasiveness of CES. The unique feature of the CES is the rapidity of expression of cellular neoplasia and interpretation of the simulated tumor in 4 days as a simulated fibrosarcoma. This system represents a system that can be used to evaluate human carcinogens in vitro in 6-10 weeks.

INTRODUCTION

Recently, we reported on the transformation of human cells to a stage of anchorage independent growth (1) and neoplasia, (2). Neoplasia was evaluated in nude mice. The route of administration was by two different routes; one by injection of transformed cells subcutaneously into the subclavicle region, (1); second, by injection of a "bolus" of cells intracranially, (3). In the case of the subcutaneous route of injection 4 1/2-6 weeks must elapsed before we could label the take as positive. Unequivocal, identification of the tumor was accomplished following excision of the tumor followed by histopathological examination requiring an additional extended period of time.

Treated cells injected intracranially into the frontal parietal sinus of the mouse killed the mice within 30-45 days. This has been described as "mouse lethality," (3). The procedure described here presents data on an in vitro procedure that can rapidly assay for neoplasia within 3 days therefore eliminating the requirement of waiting 1-1 1/2 years until the sham intracranial or subcutaneous injected control mice die of natural causes.

MATERIALS AND METHODS

Cell Culture:

Primary fibroblast cultures of foreskin tissue were prepared in the following manner: Human foreskin tissue was minced into 2mm segments in Minimum Essential Medium - Hanks' balanced salt medium (MEM-HBSS) containing 25mm Hepes buffer at pH 7.2. The tissue was rinsed three times in this medium and the tissue fragments transferred to 20 ml of MEM-HBSS supplemented with 20% FBS (Sterile Systems, Logan Utah) ,CM. and containing 0.1% collogenase (115 U per mg 4197 CLS Worthington Biochemical Corp.; Freehold, New Jersey). After dispersion of the tissue at 37° in a 4% CO_2-enriched air atmosphere for 6-8hrs, the cells were recovered by centrifugation at 650xg for 7 minutes at 12°C. The cell pellet was washed twice with CM and the final cell suspension seeded into a 75 cm^2 flask. The cell populations was allowed to attach for 72 hrs and refed once.

On day 5 following seeding the fibroblasts were selectively detached with 0.1% trypsin, (4). The fibroblast were then seeded into CM and prepared for treatment with carcinogen.

Suspension of Chemical Carcinogen and Insulin

The treatment process requires that the carcinogens be solvated in a prescribed manner to be effective in the treatment regimen. The carcinogens either aflatoxin B_1, β-propiolactone methylazoxymethanol acetate, benzo pyrene diol epoxide I (anti) or tetrahydrodimethylbenzanthracene, (AfB_1, B-Pl, MAMA, B(a)P, BPDEI or THDMBA respectively) were solvated in Spectrar acetone under red light or gold under

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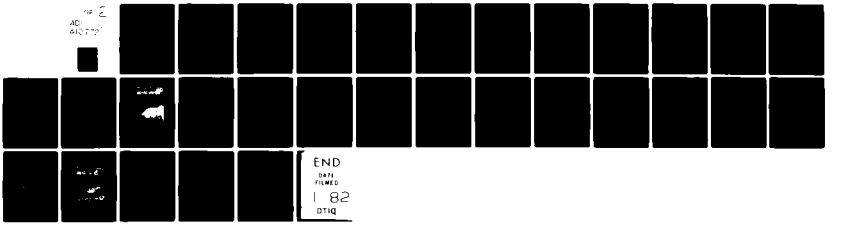

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argon. These stock solutions were made up fresh for each experiment. The working solutions at effective cytotoxic doses of 0, 25 or 50 percent were prepared from the stock solutions in complete growth medium, (CM). These solutions were added immediately to the cells as previously described, (1,2).

Transformaton Protocol

Fibroblast cell populations blocked in G1 released from the block and treated with the carcinogen in S phase of the cell cycle, (2,5) were then serially subpassaged for 16-20 population doublings (PDL) before passage through soft-agar.

All the carcinogens used in these experiments were added to the cell populations when the cells were in early S. The treated populations were exposed to the carcinogens for a few minutes (30 minutes) such as for BPDE (I) with a brief half-life or up to 12 hours for carcinogens with a long half-life, (B(a)P).

Treatment was terminated when the experimental medium was removed and the population immediately passaged at a 1:2 split ratio into CM containing additional growth supplements of 2x vitamins, 8x nonessential amino acids and 20% FBS. The treated and untreated cultures at 80-90% saturation density were passaged at 1:10 split ratio into the enriched growth medium for 20 PDL.

Growth in Soft Agar

The carcinogen treated and untreated populations at PDL 20 were seeded at 50,000 cells/25cm^2 area in 2ml of 0.33% soft agar prepared in Dulbecco's Lo Cal medium (Biolabs Northbrook, Ill.) (6,7). The cultures were incubated in a 4% carbon dioxide enriched air atmosphere and refed every 3-5 days. The incidence of colony formation was determined and after 3 weeks the colonies were removed and seeded into 75 cm^2 flasks containing CM.

These populations were then prepared for either nude mice injection or seeding on chick embryonic skins (CES) in vitro.

Evaluation of Neoplasia

The treated populations that exhibited anchorage independent growth were evaluated in 6 week old nude mice, (Sprague-Dawley, Madison, WI). The untreated cell populations would not passage through soft agar; therefore, companion untreated cultures that had not passaged through soft agar were evaluated in the nude mice (8) and on CES. The mice were irradiated with 450 RAD (from a ^{137} Cs source) 48 hrs prior to subcutaneous injection of 5x10^6 treated or control cell populations, (6). After 6 weeks the tumors were counted and the incidence of tumor formation recorded. The lack of tumor formation in injected mice was not recorded until the mice died (1-1 1/2 years).

These same treated and untreated cell populations were seeded onto CES, in vitro. The CES organ cultures were modified in the following manner to optimize the frequency of success for a rapid evaluation of cellular neoplasia, (9). We (10) have

successfully applied this technique to evaluation of carcinogen induced epithelial cell neoplasia. Growth of the treated epithelial cells into the CES simulated squamous cell carcinomas.

Fertile eggs were incubated 9 to 10 days in a humidified egg incubator, (Humidair Incubator Co., New Madison, Ohio). The embryos were removed and the skin removed from the embryo. These pieces of skin were then placed on agar bases containing 10 parts of 1% agar (Bacto-agar) in Earle's balanced salt solution without bicarbonate. This preparation also contained 4 parts FBS and 4 parts chick embryo extract, (9). The enriched agar base was poured over a steel grid implanted into the agar base. A 6-8 mm diameter section of skin was layered onto the base dermis-side up. A sterile glass ring 2 mm in thickness - 8 mm in diameter was laid over the skin. Populations from untreated and treated cell populations, containing 10^5 cells were suspended in 0.025 ml of CM+20% FBS and seeded into the rings. The dishes were then incubated in a 4% CO_2 humidified enriched air atmosphere at 37°C. On day 2 an additional 0.015 ml of CM 20% FBS was added to the rings. On day 4 the system was fixed in Bouin's solution, embedded in parafin stained with hematoxylin and eosin and evaluated by light microscopy.

Results

There is variation in cytotoxic response of treated fibroblast to treatment when the fibroblasts are obtained from different donors. The values reported in Table 1 for E.D.50 values are Mean values for 8 different wells. These concentrations were used as a carcinogenic dose for treatment of the populations in S. The compounds BPDE(I), β-propiolactone and MAMA have a relative brief half life and were left on the cells in S for 30 minutes for BPDE(I) or 3 hr for β-PL or MAMA. The chemicals, either Aflatoxin B_1 or THDMBA were left on the cells for 12 hrs.

These cell populations treated in S were serially passaged for 16 PDL prior to seeding the populations into soft agar, Table 1. All of the populations treated with each carcinogen exhibited colony formation when seeded into soft agar.

Once the protocol for transformation has been completed and abnormal colonies identified the cells were serially passaged. These cultures were continually passaged for another 16 PDL. At that time the populations were seeded into soft agar. We have observed formation from 10-900 colonies -10^{-5} cells, (Table 1) dependent upon the carcinogen treatment. Aflatoxin B_1 transformed cells produced 10 colonies per 10^5 treated cells seeded in soft agar while methylazoxy methanol acetate treated cells produced 900 cells per 10^5 treated cells. These colonies were removed, pooled and reseeded in culture. After 10 PDL, $5x10^6$ cells were injected subcutaneously into nude mice. Tumor takes were counted 4 weeks later. In three out of five transformed cell populations exhibiting anchorage independent growth in soft agar, formed tumors in nude mice, (Table 1). Tumor incidence ranges from 2 mice giving rise to undifferentiated mesenchymal tumors following the reception of $5x10^6$ methyl azoxy methanol acetate per 16 receiving the inoculum, to 8/14 receiving the aflatoxin treated cells. The nude

mice receiving treated cells were not scored as negative until 1 year later. The nude mice under our environmental conditions lived for 1-1½ years. The same untreated (Fig. 1-A and Treated (Fig. 1-B), populations that were injected into the nude mice were seeded on the CES (Fig. 1 A & B). Five micron thick hematoxylin and eosin stained sections were examined, (Fig. 1-B). The transformed cells penetrated into the layers of the CES, in many cases complete interruption of 8-10 layers of chick cells were observed. The presence of mitotic figures in the invading transformed cell populations was observed. Tumors simulated fibrosarcomas. The invasiveness of these proliferating transformed human fibroblast populations implies malignancy but are not to be compared to metases, (Fig. 1-B). We want to imply that these chemical carcinogen transformed cells contain the ability to form localized growth (tumors) on CES. We want to state also that there is a strong correlation between the tumor incidence in nude mice, growth in soft agar and microtumor formation in CES. Untreated cell populations seeded onto CES did not penetrate the upper layer of CES, (Fig. 1-A). Moreover, transformed human nasopharyngeal and testicular carcinoma cells formed localized tumors in nude mice and invaded CES. The chemically transformed populations from all 5 carcinogen treated populations (Table 1) were designated as fibrosarcomas.

These colonies were removed from the soft-agar and reseeded in culture. After 10 PDL $5x10^6$ cells were injected subcutaneously into nude mice. Tumor takes were counted 4 weeks later. In three out of the 5 transformed cell populations exhibiting anchorage independent growth in soft agar formed tumors in nude mice. The nude mice receiving treated cells were not scored as negative until 1 year later. The nude mice under our environmental conditions live for 1-1 1/2 years. Both treated and untreated

populations were also seeded onto CES and 3 days later the skin explants were removed, fixed, embedded in parafin, stained with hematoxylin and eosin, sectioned into 5 M thick sections and examined by a histopathologist, (Fig. 1-B).

On all of the untreated CES organ explants the normal untreated foreskin fibroblast populations did not proliferate, (Fig. 1-B).

The transformed cell populations from all the 5 carcinogen treated populations invaded the dermal layers of CES in 3 days, (Fig. 1-A). The tumors contained mitotic figures and were designated as fibrosarcomas.

DISCUSSION

In recent investigations we have been able to evaluate transformed human cell populations for their anchorage - independent - growth in a semi-solid medium (1) or for their tumor potential in nude mice, (2). Moreover, it was interesting to note that in each case the tumors produced by chemical carcinogen transformed cell populations that exhibited anchorage-independent - growth characteristics were classified as undifferentiated mesenchymal tumors (1,2) when evaluated in nude mice while FeSV transformed cell populations induced tumors were classified as fibromas (11), and physical carcinogen ($U.V_{254nm}$ or ^{137}Cs) induced transformed cell populations (7) were classified as myxofibromas. These evaluations of neoplasia in the nude mouse took several weeks to one year, (1,2,6). With CES the evaluations of cellular neoplasia can be completed within 4 days following seeding of the cells onto the dermis of the CES. We have not observed invasion of the CES by untreated cell populations. In every CES that receive human nasopharyngeal carcinoman or testicular carcinoma cells when tested they always formed tumors in nude mice and invaded the CES. With both negative and positive control populations we concluded that the CES system can be effectively used in vitro to evaluate carcinogen induced neoplasia of diploid human cells to a neoplastic stage.

With the current technology to transform human cells in vitro with physical carcinogens and chemical carcinogens we can now evaluate the neoplastic potential of these cells on CES in vitro with a high degree of reliability and reproducibility and within a reasonable period of time from induction to neoplasia of 6-10 weeks instead of 1-1 1/2 years. Recently, we have adapted this assay to evaluating chemically transformed human epithelial foreskin populations, in vitro for evidence of cellular neoplasia. The system lends itself to a rapid inexpensive assay that has a high correlation with growth of the treated cells in soft agar and growth of transformed cells in nude mice.

ACKNOWLEDGEMENTS

We would like to acknowledge the professional assistance of Dr. Stephen Weisbrode, a pathologist for his interpretation of the tumors. This work was supported in part by AFSOR F49620-80 and NIH-NCI R01-CA-25907

LEGEND TABLE 1

a) The selection of these compounds was based upon prior knowledge that they represent a cross-section of compounds requiring no activation . The compounds Aflatoxin B_1, Benzo (a) pyrene diol epoxide-I, were furnished by the NCI chemical repository I.I.T.R.I.; β-propiolactone was a generous gift from Dr. Joseph DiPaolo, DCCP, NCI. Methyl azoxy methanol acetate was a generous gift from Ms. Marilyn George, Department of Defense, A.F.S.O.R. Wright Patterson Airforce Base Dayton, Ohio; 1,2,3,4 tetrahydro-7,12 dimethylbenz(a)nthracene was synthesized and characterized here at O.S.U. by Dr. Donald Witiak et al*. This compound is the reduced A-ring analogue of 7,12 dimethyl benz(a)anthracene, (7,12 DMBA).

b) These concentrations were selected due to their individual effects on cell proliferation as measured by alternation in cloning of cell populations at a low cell density of 1000 cells 25cm^2, Milo, G., et al In Vitro In Press (1981). The concentration used here represent 50% cytotoxic doses.

c) Frequency of colony growth in soft agar is described as the formation of colonies of 50 cells or more when fifty-thousand cells at 20 PDL were seeded into 2 ml of 0.33% agar supplemented with LoCal and 20% FBS, overlaid over a 2.0% agar base suplemented with RPMI-1629 and 20% FBS in a 25 cm^2 well. The colonies were counted after 28 days. The frequency of growth is expressed as the number of colonies formed per 10^5 cells.

d) The tumor incidence is expressed as: the numerator value is the number of mice giving rise to tumors 0.8 - 1.2 cm in size at 4-6 weeks after injection of $5x10^6$ cells per animal subcutaneously and the denominator is the total number of nude mice injected. All animals used in these experiments were 5-6 weeks of age and preirradiated with 450 RADS whole body irradiation 24-48 hrs prior to injection. All animals were housed under cyclic light-dark cycle over a 12-12 hr cycle. The lighting in the animal room was a GE gold fluorescent light.

e) The incidence of invasiveness of chick embryonic skin organ (CES) in vitro was measured on the 4th day, i.e., 3 full days following seeding of the cells on CES. Treated cell populations that exhibited anchorage - independent -growth and untreated populations were examined on CES. The incidence of invasiveness is expressed as: the numerator value is the number of CES positive for the presence of invading human transformed cells seeded per total number of CES seeded. These figures represent values for 3-4 replicates. Out of 3 to 4 slides per CES, we observed that at least 1 out of 3 slides were positive for all populations examined.

Anchorage Independent growth, Tumor formation in nude mice and Cellular Neoplasia of Carcinogen Treated Human Foreskin Fibroblasts In Vitro

TABLE 1

Compound[a)]	Concentration (µg/ml)[b)]	Frequency Colony Growth In Soft Agar	Tumor Incidence[d)]	Incidence of Invasivenss CES[e)]
Aflatoxin B_1	10	10	8/14	100
β-propiolactone	13	14	3/4	100
Benzo (a) Pyrene diol epoxide (I)	0.1	26	N.D.	100
Tetrahydro-7,12 dimethyl benz(a)anthracene	1.0	84	N.D.	100
Methyl azoxy methanol acetate	3.6	900	2/16	100

N.D. - not done

REFERENCES

1. Milo, G., and Di Paolo, J., Neoplastic transformation of human diploid cells in vitro after chemical carcinogen treatment. Nature, 275:130-132; 1978

2. Milo, G., and Di Paolo, J., Presensitization of human cells with extrinsic signals to induced chemical carcinogenesis. Intl. J. of Cancer 26:805-812; 1980.

3. Greiner, J., Evans, C., and Di Paolo, J., Carcinogen induced neoplastic transformation of human MRC-5 cells. Carcinogenesis In Press, 1981

4. Milo, G., Ackerman, A., and Noyes, I., Growth and Ultrastructual Characterization of proliferating human keratinocytes in vitro without added extrinsic factors. In Vitro 16:20-30; 1980

5. Donahoe, J., Noyes, I., Weisbrode, S., Schumm, D., and Milo G., A suitable rapid in vitro organ culture system to evaluate neoplasia exhibited by carcinogen transformed human fibroblasts, Proc. Amer. Assoc. Cancer Res. 22:117; 1981

6. Milo, G., Oldham, J., Zimmerman, R., Hatch, G., and Weisbrode, S., Characterization of human cells transformed by chemical and physical carcinogens, in vitro. In Vitro In Press 1981

7. Milo, G., Weisbrode, S., Zimmerman, R., and McCloskey, J., Ultraviolet induced neoplastic transformation of normal human cells, In Vitro. Chem. Biol. Int In Press 1981

8. Giovanella, B., Nilsson, K., Zech, L., Klein, G., and Stehlin, J., Growth of diploid, Epstein-Barr virus carrying human lymphoblastoid cell lines heterotransplanted into nude mice under immunologically privileged conditions. Intl. J. Cancer 24:103-113; 1979.

9. Noguechi, P., Johnson, J., O'Donnell, R., and Petriciani, J., Chick Embryonic Skin as a rapid organ culture assay for cellular neoplasia, Science 199:980-983; 1978

10. Milo, G., Noyes, I., Donahoe, J. and Weisbrode, S., Neoplastic transformation of human epithelial cells in vitro after exposure to chemical carcinogens. Cancer Res In Press 1981

11. Milo, G., Olsen, R., Weisbrode, S., and McCloskey, J., Feline Sarcoma virus induced in vitro progression from premalignant to neoplastic transformation of human diploid cells. In Vitro 16: 813-822; 1980

Legend 1. In Fig. 1-A chick embryonic (CES from 9 day old chick embryo) prepared as described in Materials and Methods section were supplanted onto a 5 ml 1% Bacto-agar base supplemented with growth factors. One hundred thousand untreated cells were seeded onto the CES and these organ cultures incubated at 37° for 4 days in a 4% CO_2 enriched air environment (x 160). These fibroblasts appeared as necrotic cells on the dermal surface of the CES.

In Fig. 1-B CES organ cultures prepared as described above received 10^5 cells of transformed cells restablished from 0.33% agar layers. After 4 days in a 4% CO_2 enriched-air environment they were evaluated. Invasion of the CES by the transformed cells is illustrated by arrows.

A

B

ACKNOWLEGEMENTS

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Carcinogenesis, Volume 2, Number 9, 1981

Short Communication

Effect of pH on the neoplastic transformation of normal human skin fibroblasts by N-hydroxy-1-napthylamine and N-hydroxy-2-naphthylamine

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Abstract

The conversion of urinary N-hydroxy arylamines to carcinogenic electrophiles under mildly acidic conditions in the bladder lumen has been proposed as an essential step in arylamine-induced urinary bladder carcinogenesis. To test the hypothesis that extracellular generation of an ultimate carcinogenic species can initiate a neoplastic event normal human fibroblasts were exposed to N-hydroxy-1- and 2-naphthylamine (N-HO-1-NA and N-HO-2-NA) at pH 5 and pH 7. With both compounds, anchorage independent growth of transformed cells in soft agar were enhanced 3- to 7-fold in the pH 5 incubations. Injection of the N-HO-1-NA and N-HO-2-NA-transformed cells into nude mice resulted in tumors in 6/8 and 2/7 animals, respectively. In a control experiment, no differences in transformation of these cells by irradiation (^{137}Cs) were observed between pH 5 and pH 7 exposures. Thus, the results are consistent with the hypothesis that ultimate carcinogenic electrophiles, generated extracellularly, can enter an intact cell and induce neoplasia. Alternatively, the possibility of a local extracellular acidic environment near the cell surface and its role in the generation of a reactive electrophile leading to urinary bladder carcinogenesis is discussed.

N-Hydroxy arylamines have been postulated as ultimate carcinogenic metabolites for the induction of urinary bladder cancer by aromatic amines and nitroarenes (1–4). The N-hydroxy derivatives enter the blad-

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[5]Abbreviations: NFS, neonatal foreskin; FBS, fetal bovine serum; PDL, population doubling; N-HO-1-NA, N-hydroxy-1-napthylamine; N-HO-2-NA, N-hydroxy-2-napthylamine; CM, complete growth medium; DNA, deoxyribonucleic acid; HEPES; N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; S, scheduled DNA synthesis of the cell cycle; ID_{50}, effective dosage in reducing the cloning efficiency to 50% of the control.

der lumen as urinary N-glucuronide conjugates which hydrolyze to the free N-hydroxy arylamines, particularly under the mildly acidic conditions normally found in human and canine urines (2). Under the same acidic conditions, the N-hydroxy metabolites may be chemically converted to reactive electrophiles; and the subsequent binding of these urinary electrophiles to urothelial nucleic acids has been proposed to be a critical event in arylamine-induced bladder carcinogenesis (2–4).

Previous studies have shown that the *in vitro* binding of N-hydroxy-1-naphthylamine (N-HO-1-NA)* or N-hydroxy-2-naphthylamine (N-HO-2-NA) to calf thymus DNA increases 20-fold as incubation conditions are altered from a neutral to a slightly acidic pH (2,5,6). N-HO-1-NA reacted with the DNA exclusively at the O^6 atom of deoxyguanosine, while N-HO-2-NA yielded adducts at the N^6 position of deoxyadenosine and at the N7 and C8 atoms of deoxyguanosine (5,6).

If this mechanism is important in the initiation of urinary bladder carcinogenesis, then the passage of these highly reactive electrophiles from the extracellular environment (e.g., bladder lumen) into the intact target cell (e.g. urothelium) must occur. To test this hypothesis, we examined the effect of extracellular pH on the ability of N-HO-1-NA and N-HO-2-NA to induce the transformation of normal human neonatal skin (NSF) fibroblasts. This system, which has been extensively characterized (7–11), was selected as an appropriate model for evaluating ultimate urinary bladder carcinogens since N-hydroxy arylamines not only induce carcinomas when instilled urethrally into the bladder lumen (12), but also induce sarcomas at sites of s.c. or i.p. application (12–15; and E.C.Miller, F.F.Kadlubar, J.A.Scribner, and J.A.Miller, unpublished studies).

The methods of transformation of normal human fibroblasts have been described in detail elsewhere (7,8). Primary cultures of NFS fibroblasts (9,10) were grown in Eagles' minimal essential medium with Hank's balanced salt solution – 25.0 mM HEPES buffer (Grand Island Biological Company, Grand Island, NY), pH 7.2, supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids (MA Bioproducts, Bethesda, MD) 2.0 mM glutamine (MA Bioproducts), 0.2% sodium bicarbonate, 5.0 μg/ml gentamycin, and 10% fetal bovine serum (FBS, Rehatuim, Reheis Chemical Company, Phoenix, AZ), hereafter referred to as complete medium (CM). Cultures were maintained at 37°C in a 4% CO_2, high humidity environment. The cells were passaged to population doubling (PDL) 5, and when at confluent-density were removed by trypsinization (0.1%) and reseeded into 75 cm^2 tissue culture flasks at a 1:4 split ratio in [illegible]

All handling of the test compounds was conducted under gold light (G.E. Ultrabright Tubes) or in the dark. As indicated below, most manipulations were carried out in an inert atmosphere in order to ensure compound stability. N-HO-1-NA and N-HO-2-NA were synthesized by the method of Willstatter and Kubli (16). The carcinogens were dissolved in acetone (Spectrar grade, Mallinckrodt, Inc., Paris, KY), under argon, at a concentration of 1.0 mg/ml just prior to use. The treatment medium consisted of CM minus FBS (pH 7), and it was adjusted to pH 5.0 and pH 7.0 with HCl and NaOH, respectively, deaerated by aspiration, and purged with argon.

The cultures in early S were washed with the appropriate treatment medium (pH 5.0 or 7.0) and the flasks were gassed with argon. Just prior to the addition to the cultures, N-HO-1-NA and N-HO-2-NA were added to the treatment medium at concentrations of 2.0 μg/ml, which was determined to be the ED_{50} (dose effective in reducing the cloning efficiency to 50% of the control; ref. 8) for these compounds on NFS fibroblasts at both pH 5 and 7. The comparative viability of each cell population was the same for pH 5.0 and 7.0 at an ED_{50} cytotoxic value. The acetone concentration was 0.2%, the same concentration to which control cultures at pH 5.0 and pH 7.0 were exposed. Once the carcinogens were added, the medium was immediately added to the cultures (15 ml flask), and they were placed at 37°C for 15 min. In separate, 15 min incubations without cells, the recovery (2) of N-HO-1-NA was 97 ± 2% at pH 5.0 and 97 ± 4% at pH 7.0; for N-HO-2-NA, the values were 97 ± 3% and 93 ± 2%, respectively (n = 3). Following the exposure period, the carcinogens were removed, the cultures were washed with Cm, and then they were fed with 8x medium (CM supplemented with 8x nonessential amino acids, 2x vitamins, and 20% FBS). When the cells reached near confluent density (80–90%) the cultures were passaged at 1:10 split ratios in 8x medium until they reached PDL 20. At this point, they were seeded into 0.33% agar and the frequency of colony formation in soft agar was determined (8). Monolayer cultures were reestablished from colonies removed from the agar, and the cells were subsequently injected s.c. into preirradiated nude mice in order to determine the neoplastic potential of the transformed cells (7,8).

The results of the transformation experiments are presented in Table I. The studies were done on 2 separate occasions with comparable results. Treatment with N-HO-1-NA at pH 5.0 induced colony formation in soft agar at frequencies of 6.9 and 3.3 times that induced at pH 7.0 in experiments 1 and 2, respectively. Similarly, treatment with N-HO-2-NA at pH 5.0 induced colony formation frequencies in soft agar of 4.1 and 4.0 times that induced at pH 7.0 in experiments 1

In order to determine the tumorigenic properties of the transformed cells, monolayer cultures were re-established from boluses induced by N-HO-1-NA and N-HO-2-NA at pH 5.0 in experiment 2. One of 8 mice developed a tumor following the injection of N-HO-1-NA transformed cells, and 2 of 7 mice developed tumors following the injection of N-HO-2-NA tranformed cells. Tumor development following the injection of normal fibroblasts into the nude mouse has never been seen in our laboratory. As described previously (7,8,11), morphologically transformed cells ($\sim$5 x 10^6) were injected s.c. into the interscapular region of 5–6 week old nude mice. By 4 weeks, tumors were 0.8–1.2 cm. They were then enumerated and histopathologic studies of the masses confirmed them as neoplastic, undifferentiated mesenchymal tumors with varying abnormal karyotypes (7,8,11)).

As a control for the possible effects of pH on the sensitivity of these cells for transformation, 5 μg/ml of aflatoxin B_1 was also added to the companion fibroblast cultures at pH 7.0 and 5.0 as described above. After 20 PDL, the treated cell populations were seeded in 0.33% agar. The frequencies of colony formation in each treatment at pH 5.0 and 7.0 for 2 experiments was comparable: 13/16 colonies/10^5 cells at pH 5.0 and 9–13 colonies/10^5 cells for pH 7.0. Moreover, the growth rate of the pH 5.0 and 7.0 treated populations was not appreciably different. These aflatoxin-treated cells in two experiments also formed tumors in nude mice at a frequency of 4 or 6 tumors/7 mice for treated cells at pH 5.0 and 3 tumors/8 mice at pH 7.0.

Repeated attempts were also made to estimate the covalent binding of the carcinogenic N-hydroxy arylamines to the DNA of NFS cells after the 15 min exposure period using [5,6,7,8-^{3}H]N-HO-2-NA (373 mCi/mmol) or [3-^{3}H]N-HO-1-NA (181 mCi/mmol), obtained from Dr R.Roth, Midwest Research Institute, Kansas City, MO. Although the DNA, which was isolated by hydroxylapatite chromatography (17), consistently contained low levels of radioactivity (200–600 d.p.m. corresponding 0.3–1.0 naphthyl residues/10^6 nucleotides), these amounts were too low to permit quantitative comparisons between pH 5.0 and pH 7.0 incubations.

Thus, the results obtained in this study clearly demonstrate that acidic pH substantially increases the frequency of transformation of normal cells exposed to carcinogenic N-hydroxy arylamines. The possibility that this is simply due to the increased uptake of the N-hydroxy compounds at pH 5 seems unlikely since no similar increase was noted above for aflatoxin B_1. In addition, when N-HO-1-NA and N-HO-2-NA were instilled directly into the urinary bladder in pH 5- and pH 7-buffered solutions, no differences in their absorption or entry into the circulation were observed (18). However, since N-hydroxy arylamines partially decompose to electrophiles under acidic conditions, the data indicate that these ultimate carcinogenic species may enter the protective environment of the cell nucl

nitrosamines and nitrosamides (19,20), indicate a similar effect of pH on their S9-dependent mutagenicity in *Salmonella typhimurium*. Although it was suggested that cells may become sensitized to mutagens at acidic pH, it is also likely that metabolically formed electrophiles, presumably alkyldiazonium ions, might be expected to have greater stability at acidic pH and thus enter the bacterial cell and result in a mutation. Therefore, it is reasonable to assume that the carcinogenic N-hydroxy arylamines and their electrophilic derivatives formed in the bladder lumen or at the cell surface under acidic conditions, can alter the urothelial cell by reaction with critical informational macromolecules, thereby initiating the neoplastic process.

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References

1. Clayson,D.B., and Garner,R.C. (1976), Carcinogenic aromatic amines and related compounds, in Searle,C.E., (ed.), *Chemical Carcinogens*, ACS Monography 173, American Chemical Society, Washington, DC, pp. 366-461.
2. Kadlubar,F.F., Miller,J.A., and Miller,E.C. (1977), Hepatic microsomal N-glucuronidation and nucleic acid binding of N-hydroxy arylamines in relation to urinary bladder carcinogenesis, *Cancer Res.*, 37, 805-814.
3. Radomski,J.L., (1979), The primary aromatic amines, their biological properties, and structure-activity relationships, *Annu. Rev. Pharmacol. Toxicol.*, 19, 129-157.
4. Kadlubar,F.F., Unruh,L.E., Flammang,T.J., Sparks,D., Ruchirawat,R.K., and Mulder,G.J. (1981), Alteration of urinary levels of the carcinogen, N-hydroxy-2-naphthylamine, and its N-glucuronide in the rat by control of urinary pH, inhibition of metabolic sulfation, and changes in biliary excretion, *Chem.-Biol. Interactions*, 33, 129-147.
5. Kadlubar,F.F., Miller,J.A., and Miller,E.C. (1978), Guanyl O^6-arylamination and O^6-arylation of DNA by the carcinogen N-hydroxy-2-naphthylamine, *Cancer Res.*, 38, 3628-3638.
6. Kadlubar,F.F., Unruh,L.E., Beland,F.A., Straub,K.M., and Evans,F.E. (1980), *In vitro* reaction of the carcinogen, N-hydroxy-2-naphthylamine, with DNA at the C-8 and N^2 atoms of guanine and at the N^6 atom of adenine, *Carcinogenesis*, 1, 139-150.
7. Milo,G.E., and Dipaolo,J. (1978), Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment, *Nature*, 275, 130-132.
8. Milo,G.E., Oldham,J.W., Zimmerman,R., Hatch,G.G., and Weinhouse,S.A. (1981), Characterization of human cells transformed by chemical and physical carcinogens *in vitro*, *In Vitro*, in press.
9. Riesner,D., McMichael,T., Berno,J., and Milo,G. (1976), Processing of human tissue to establish primary cultures *in vitro*, *Tissue Culture Assoc. Lab. Manual*, 2, 273-276.
10. Oldham,J.W., Allred,L.E., Milo,G.E., Kindig,O., and Capen,C.C. (1980), The toxicological evaluation of the mycotoxins T-2 and T-2 tetraol using normal human fibroblasts *in vitro*, *Toxicol. Appl. Pharmacol.*, 52, 159-168.
11. Milo,G., and DiPaolo,J. (1980), Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis, *Int. J. Cancer*, 26, 805-812.
12. Radomski,J.L., Brill,E., Deichmann,W.B., and Glass,E.M. (1972), Carcinogenicity testing of N-hydroxy and other decomposition products of 1- and 2-naphthylamine, *Cancer Res.*, 31, 1461-1467.
13. Dooley,K.L., Beland,F.A., Heath,J.E., and Kadlubar,F.F. (1980), Focal carcinogenicity of N-hydroxy-1- and 2-naphthylamine in relation with rates of absorption and DNA binding, *Proc. Am. Assoc. Cancer Res.*, 22, 335.
14. Boyland,E., Duke,C.E., and Grover,P.L. (1963), Carcino-

18 Oglesby,L.A., Flammang,T.J., Tullis,D.L., and Kadlubar,F.F. (1980). Rapid absorption, distribution, and excretion of carcinogenic N-hydroxy arylamines after direct urethral instillation into the rat urinary bladder. *Carcinogenesis*, 2, 15-20.

19 Prattenplan,J.B. (1980). Enhanced mutagenic activities of N-nitroso compounds in weakly acidic media. *Carcinogenesis*, 1, 69-84.

20 Neetshi,T., and Hayatsa,H. (1980), The pH-dependent response of *Salmonella typhimurium* TA100 to mutagenic N-nitrosamines, *Mutat Res.*, 79, 223-230.

Table I

Effect of treatment pH on colony formation in soft agar by N-hydroxy napthylamines

Treatment[a]	Colony formation[b] pH 5.0	pH 7.0	pH 5.0/ pH 7.0
N-HO-1-NA (Exp. #1)	131 ± 7	19 ± 3	6.9
(Exp. #2)	30 ± 3	9 ± 2	3.3
N-HO-2-NA (Exp. #1)	95 ± 6	23 ± 4	4.1
(Exp. #2)	28 ± 3	7 ± 3	4.0
Aflatoxin B_1 (Exp. #)	13 ± 2	9 ± 3	1.4
(Exp. #2)	16 ± 4	13 ± 2	1.2
Control	0	0	—

[a] Doses used were 2.0 μg/ml for N-HO-1-NA and N-HO-2-NA, and 5 μg/ml for aflatoxin B_1. Controls (pH 5.0 and pH 7.0) were exposed to 1.2% acetone.

[b] Frequency of growth in soft agar (0.33%), expressed as the number of colonies per 100,000 cells. Each well received 50,000 treated cells in 2 ml of soft agar seeded over a 5 ml 2% agar base (8,11). For each value given, a s.d. is expressed and is based on results from eight wells (n = 8).

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CHARACTERISTICS OF BENZO[*a*]PYRENE AND A-RING REDUCED 7,12-DIMETHYL BENZ[*a*]ANTHRACENE INDUCED NEOPLASTIC TRANSFORMATION OF HUMAN CELLS IN VITRO*

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SUMMARY

The polynuclear aromatic hydrocarbons (PAH) benzo[*a*]pyrene (BP) and the A-ring reduced analogue of 7,12-dimethylbenz[*a*]anthracene (DMBA), 1,2,3,4-tetrahydro-7,12-dimethylbenz[*a*]anthracene (TH-DMBA) are carcinogenic to human cells. The unsaturated PAH, DMBA exhibits no carcinogenic activity on human cells as measured by growth in soft agar. The TH-DMBA and BP treated cells exhibit a colony frequency in soft agar of 84 and 86, respectively. These anchorage independent cells, when seeded on the chick embryonic skin (CES) organ cultures, are invasive and form a fibrosarcoma. It is highly unlikely that TH-DMBA, which does not contain an aromatic A-ring, can undergo metabolism in human cells in culture to form a bay region 3,4-dihydrodiol-1,2-epoxide. These results suggest that an alternate mechanism for the induction of carcinogenesis is appropriate to explain the absence of bay region diol-epoxide metabolite as the ultimate form of the carcinogen in TH-DMBA induced carcinogenesis in human diploid cells.

INTRODUCTION

Metabolic conversion of PAH such as BP and DMBA has been shown to precede an expression of their toxic, mutagenic or carcinogenic activities [1,2,5,6,17,21]. Our previous studies with BP have indicated that this car-

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cinogen can induce neoplastic transformation in proliferating human skin fibroblast cells when added during the S phase of the cell cycle [4, Milo et al., unpublished data]. Although DMBA can induce a carcinogenic event in rodent cells in culture [12], this PAH does not induce such an event in proliferating human skin fibroblast cells in culture [4].

Previous results from our laboratory have indicated that BP is taken up and initially bound to a cytoplasmic lipoprotein complex present in human skin fibroblast cells before being transported to the nucleus [4,19, Tejwani, unpublished data]. DMBA, on the other hand, is randomly dispersed throughout these cells and is not bound to the cytoplasmic lipoprotein complex [4, Tejwani, unpublished data]. These differences may in part explain the induction of neoplastic transformation in the normal human skin fibroblast cells by BP and not by DMBA.

The bay region diol-epoxide of DMBA, the 3,4-dihydrodiol-1,2-epoxide, has been proposed to be the major metabolite responsible for the mutagenic and carcinogenic activities of this PAH in rodent cells [3,18]. Consistent with this proposal, DMBA exhibits mutagenicity in the Ames assay only in the presence of a microsomal activation system [7].

The observation that the PAH TH-DMBA was mutagenic in the absence or presence of a microsomal activation system, using 3 strains (TA1537, TA98, TA100) of *Salmonella typhimurium* [7], provided impetus to study this A-ring reduced analogue for carcinogenic activity, using human cells. Since TH-DMBA cannot be expected to yield a bay region diol-epoxide unless it was first oxidized (aromatized) to DMBA, this A-ring reduced analogue should serve as a useful probe for investigating alternate mechanisms of transformation.

In our laboratory, we have defined several indices of human fibroblast cell transformation in response to a variety of chemical carcinogens. These include morphological changes, extended life span, growth in culture conditions toxic for untreated normal cells, increase in lectin agglutinability and alteration in cellular prostaglandin levels [10]. The anchorage-independent growth of transformed cells in soft agar has been the most consistent and reliable indicator of tumor production in athymic nude mice and on the CES organ culture system.

In this report, data are presented on the carcinogenicity of TH-DMBA in human neonatal foreskin (HNF) fibroblast cells in culture.

MATERIALS AND METHODS

Chemicals

DMBA was purchased from Eastman Chemical Company and BP was received from the National Cancer Institute Repository. TH-DMBA was synthesized by a method previously described [7,20]. The hydrocarbon was purified on a Spherisorb ODS 5 μm column (4.5 mm × 25 cm) using a linear gradient of 25—100% methanol for 1 h. All solvents were of reagent or analytical grade.

Cell cultures and treatment of cells with PAH

Primary HNF cell cultures were established as described previously [14]. Randomly proliferating cell populations, derived from human foreskin tissues dispersed with collagenase, were passaged in complete growth medium (CM) composed of Eagle's minimum essential medium: 25 mM Hepes buffer (pH 7.2) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 50 μg/ml gentocin, 0.2% sodium bicarbonate and 10% fetal bovine serum (FBS) in a 4% CO_2-enriched air atmosphere at 37°C. HNF cells passaged at a 1 : 4 split ratio reached confluency in 4 days.

Preconfluent (70—80%) logarithmically growing HNF cell populations between population doubling (PDL) 4 to 5 were blocked at the G_1/S phase of the cell cycle by feeding the cultures with non-proliferating Dulbecco's modified Eagle's medium supplemented with 50 μg/ml gentocin, 1 mM sodium pyruvate and 10% dialyzed FBS [8—10]. Twenty four hours after seeding, when the mitotic index was 0.1% to 0%, the non-proliferating medium was removed and the cell cultures were re-fed with CM supplemented with 0.5 U/ml insulin. Thirty four hours after seeding, the synchronized cell cultures were treated with 3.2 μg/ml of BP, 1 μg/ml of DMBA or 0.5 μg/ml of TH-DMBA in acetone. Cell cultures to which an equal volume of acetone was added served as controls. The carcinogen was allowed to remain in contact with the cells during the S phase of the cell cycle which was 8.2 h long [8—10].

Selection of transformed cell populations

Forty eight hours after seeding, the cell cultures were passaged at a 1 : 2 split ratio into CM supplemented with 2× essential vitamins, 8× non-essential amino acids and 20% FBS (selection medium). The cell populations were serially passaged at 1 : 10 split ratio into the selection medium [8—10]. After 16 PDL, the cell populations were seeded into soft agar.

Anchorage independent growth

The treated and control cell populations were trypsinised and seeded at 50,000 cells/25 cm^2 well in 2 ml of 0.33% soft agar and Dulbecco's LoCal medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 50 μg/ml gentocin, 0.2% sodium bicarbonate, 1× essential vitamins, 1× essential amino acids and 20% FBS. The cells were layered over 5 ml of a 2% agar base prepared in RPMI 1629 medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 50 μg/ml gentocin, 0.2% sodium bicarbonate, 1× essential vitamins, 1× essential amino acids and 20% FBS [8—10]. The soft agar cultures were incubated at 37°C in a 4% CO_2-enriched air atmosphere. Three weeks later, the colonies were removed with a tuberculin syringe (20 gauge needle) and seeded into the selection medium. After attachment and sufficient growth, the cell cultures were trypsinized and re-seeded in order to evenly distribute the cells from the monolayer colonies.

The cell cultures were passaged once at a 1 : 4 split ratio and then seeded on the CES to evaluate neoplasia.

Neoplastic transformation

The cell populations at PDL 36 were seeded on the CES organ cultures prepared from 9 to 18-day-old fertilized eggs [Milo et al., unpublished data]. The CES were layered onto an agar base containing 10 parts of 1% Agar in Earle's balanced salt solution, 4 parts of chick embryo extract and 4 parts of FBS.

Then, cells (10^5) from treated and control cultures were suspended in 0.025 ml CM supplemented with 20% FBS and seeded on the CES. Three days later, the skins were removed and fixed in Bouin's solution. Transverse sections (5 μm) were stained with hematoxylin and eosin and examined for the presence of invasive features.

RESULTS

Chemical carcinogen induced neoplastic transformation of HNF cells in culture was carried out by the procedure of Milo and DiPaolo [8,9]. Randomly proliferating low passage cell populations were seeded into amino acid deficient medium to block them at the G_1/S phase of the cell cycle. The cell populations were released from the block in presence of a growth promoter. Ten hours later, in the S phase of the cell cycle, the cell cultures were treated with BP, DMBA or TH-DMBA. Cell populations transformed by BP, DMBA or TH-DMBA were serially passaged for 16 PDL

TABLE 1

CHARACTERIZATION OF TRANSFORMED HUMAN FORESKIN FIBROBLAST CELLS THROUGH ANCHORAGE INDEPENDENT STAGE AND NEOPLASTIC STAGE OF CARCINOGENESIS

Compound	Concentration (μg/ml)[a]	Evidence of colony formation in soft agar[b]	Incidence of tumor formation in CES[c]
BP	3.2	86	1/1[d]
DMBA	1	0	N.D.
TH-DMBA	0.5	84	1/1

[a] The concentration of each of these compounds was appropriately selected from results of previous work [4].

[b] Four wells (25 cm^2/well) were seeded with 50,000 cells/well in 0.33% soft agar supplemented with growth medium, over a 2% agar base [8—10]. The values for colony formation reported here were the number of colonies formed per 10^3 cells seeded into soft agar [8—10].

[c] These values reported here were for 4 CES seeded with 10^4 cells/CES and evaluated 3 days later. Normal untreated cells did not invade the tissue.

[d] BP treated cells were also evaluated for tumor formation in nude mice [4]. The treated cells were positive and formed tumors.

and seeded into soft agar to evaluate anchorage independent growth. BP (3.2 μg/ml) treated cells exhibited anchorage independent growth with a frequency of colony formation of $86/10^5$ cells (Table 1). Although DMBA (1 μg/ml) did not transform HNF cells in culture as measured by anchorage independent growth, cells transformed by TH-DMBA (0.5 μg/ml) grew to spherical colonies 10—12 days after seeding on soft agar (Fig. 1), with a frequency of colony formation of $84/10^5$ cells (Table 1).

The BP and TH-DMBA treated cell populations exhibiting anchorage independent growth were seeded onto the CES to evaluate neoplasia. These transformed cell populations were invasive on the CES and produced fibrosarcomas (Fig. 2). The tumors were designated as fibrosarcomas because of the presence of mitotic figures in the tumor tissue.

Following growth in soft agar and re-seeding into monolayer cultures, BP treated cells were also evaluated for neoplasia in nude mice. Six-week-old mice received 5×10^6 cells subcutaneously. Four weeks later, the tumors were excised and submitted to histopathology for evaluation. The tumors produced were identified as fibrosarcomas.

Fig. 1. Randomly proliferating HNF cell populations blocked in the G_1 phase of the cell cycle were released from the block and subsequently treated with TH-DMBA. The cell populations were serially passaged for 16 PDL and seeded at 50,000 cells/25 cm^2 well into soft agar as described under Materials and Methods. The soft agar cultures were incubated at 37°C in a 4% CO_2-enriched air atmosphere for 3 weeks. Each colony contained 50—300 cells, 14 days following seeding into soft agar.

A

B

Fig. 2. The BP or TH-DMBA treated cell populations exhibiting anchorage independent growth were removed and serially passaged. Then, 10^4 cells suspended in 0.025 ml CM supplemented with 20% FBS were seeded on the CES [Milo et al., unpublished data]. Three days later, the CES were fixed in Bouin's solution, stained with hematoxylin and eosin and microscopically examined. (A) represents normal untreated fibroblast cells seeded on the CES at 160x magnification. (B) represents TH-DMBA treated cell populations growing on the CES at 160x magnification.

DISCUSSION

The PAH TH-DMBA was mutagenic in the Ames assay both with and without metabolic activation in 3 strains of *S. typhimurium* [7]. In fact, this A-ring reduced analogue was more mutagenic in the absence of microsomal activation using the plasmid deficient strain TA1537 [7]. TA1538 and the missense tester strain TA1535, which are relatively insensitive to DMBA mutagenesis were not mutated by TH-DMBA [7]. Nonetheless, DMBA required metabolic activation by addition of the S9 microsomal fraction in order to elicit mutagenicity in strains TA1537, TA98 and TA100 [7], whereas TH-DMBA required no such metabolic activation. TH-DMBA, in the absence of the S9 fraction had mutagenic properties similar to metabolically activated DMBA. This is a marked departure from other PAH which require an activating system or chemical modification to a reactive species to effect mutagenesis in the Ames assay. Thus, even 3,4-dihydrodiol-7-methylbenz[*a*]-anthracene was non-mutagenic in the strain TA98, when cofactors required for the microsomal activation system were omitted [11].

Previous results have indicated that when HNF cells are treated with BP, a major portion of the PAH is bound to a cytoplasmic lipoprotein complex and is subsequently transported to the nucleus as the parent compound [4,19, Tejwani, unpublished data]. HPLC analysis of the BP metabolites covalently bound to DNA has indicated a low level of BP metabolism taking place in these cells, with the formation of a small amount of the BP-7,8-diol-9,10-epoxide-I-deoxyguanosine adduct [Tejwani, unpublished data]. In the present study, BP also induced neoplastic transformation in the HNF cells and the treated cell populations exhibited anchorage independent growth with a frequency of colony formation of $86/10^5$ cells seeded in soft agar. These transformed cell populations were invasive on the CES and produced a fibrosarcoma.

Although DMBA did not transform HNF cells in culture as measured by anchorage independent growth, it was interesting to observe that TH-DMBA, the analogue of DMBA completely reduced in the bay region, could induce neoplastic transformation in these cells and was as potent as BP, at 1/6 the dose of BP used. This pronounced activity of TH-DMBA is similar to what would be expected in our system if we were assessing an ultimate carcinogen.

Previous reports have indicated that 1,2,3,4-tetrahydro-7-methylbenz[*a*]-anthracene, a 12-desmethyl analogue of TH-DMBA, is non-carcinogenic [16]. It is known that the 12-methyl function in DMBA provides sufficient steric interaction with the C_1-carbon—hydrogen of the A-ring to stabilize a ketone function at position 5 owing to decreased planarity of the tetracyclic system [13,14]. In TH-DMBA the C_{12}—C_1 interaction freezes the ring A in a half-chair conformation with C_2 slightly and C_3 markedly out of plane with the aromatic anthracene system. The speculative A-ring triol of DMBA, possibly arising by reaction of DNA or other macromolecular nucleophile with the proposed bay region 3,4-dihydrodiol-1,2-epoxide, is expected to have a similar conformation.

These observations question the necessity for metabolic activation in the case of TH-DMBA as a prerequisite to macromolecular binding and subsequent transformation in human cells. Work is in progress employing radiolabeled TH-DMBA to further assess this phenomenon.

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REFERENCES

1 Autrup, H., Harris, C.C., Trump, B.F. and Jeffrey, A.M. (1978) Metabolism of benzo-(a)pyrene and identification of the major benzo(a)pyrene-DNA adducts in cultured human colon. Cancer Res., 38, 3689—3696.
2 Chou, M.W. and Yang, S.K. (1978) Identification of four trans-3,4-dihydrodiol metabolites of 7,12-dimethylbenz(a)anthracene and their *in vitro* DNA-binding activities upon further metabolism. Proc. Natl. Acad. Sci. U.S.A., 75, 5466—5470.
3 Dipple, A. and Nebzydoski, J.A. (1978) Evidence for the involvement of a diol-epoxide in the binding of 7,12-dimethylbenz[a]anthracene to DNA in cells in culture Chem.-Biol. Interact., 20, 17—26.
4 Ekelman, K. and Milo, G.E. (1978) Cellular uptake, transport and macromolecular binding of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene by human cells *in vitro*. Cancer Res., 38, 3026—3032.
5 Feldman, G., Remsen, J., Wang, W. and Cerutti, P. (1980) Formation and excision of covalent DNA adducts of benzo(a)pyrene 4,5-epoxide I in human lung cells A549. Biochemistry, 19, 1095—1101.
6 Huberman, E., Chou, M.W. and Yang, S.K. (1979) Identification of 7,12-dimethylbenz-(a)anthracene metabolites that lead to mutagenesis in mammalian cells. Proc. Natl. Acad. Sci. U.S.A., 76, 862—866.
7 Inbasekaran, M.N., Witiak, D.T., Barone, K. and Loper, J.C. (1980) Synthesis and mutagenicity of A-ring reduced analogues of 7,12-dimethylbenz(a)anthracene. J. Med. Chem., 23, 278—281.
8 Milo, G.E. and DiPaolo, J. (1978) Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment. Nature (London), 275, 130—132.
9 Milo, G.E. and DiPaolo, J.A. (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis, Int. J. Cancer, 26, 805—812.
10 Milo, G.E., Oldham, J., Zimmerman, R., Hatch, G. and Weisbrode, S. (1981) Characterization of human cells transformed by chemical and physical carcinogens *in vitro*. In Vitro, in press.
11 Malaveille, C., Bartsch, H., Tierney, B., Grover, P.L. and Sims, P. (1978) Microsome-mediated mutagenicities of the dihydrodiols of 7,12-dimethylbenz(a)anthracene: high mutagenic activity of the 3,4-dihydrodiol. Biochem. Biophys. Res. Commun., 83, 1468—1473.
12 Marquardt, H., Baker, S., Tierney, B., Grover, P.L. and Sims, P. (1979) Comparison of mutagenesis and malignant transformation by dihydrodiols from benz(a)anthracene and 7,12-dimethylbenz(a)anthracene. Br. J. Cancer, 39, 540—647.
13 Newman, M.S. and Olson, D.R. (1974) A new hypothesis concerning the reactive

species in carcinogenesis by 7,12-dimethylbenz(a)anthracene. The 5-hydroxy-7,12-dimethylbenz(a)anthracene-7,12-dimethylbenz(a)anthracene-5(6H)-one equilibrium. J. Am. Chem. Soc., 96, 6207—6208.

14 Newman, M.S. and Cunico, R.F. (1972) The synthesis of 5-hydroxymethyl-, 5-acetoxymethyl- and 5-methylmercapto-7,12-dimethylbenz(a)anthracenes and of 5,7,12-trimethylbenz(a)anthracene. J. Med. Chem., 15, 323—325.

15 Riegner, D.T., Berno, M. and Milo, G.E. (1976) Processing of human tissue to establish primary cultures *in vitro*. Tissue Culture Association Lab. Manual, 2, 273—276.

16 Shear, M.J. (1938) Studies in carcinogenesis of methyl derivatives of 1:2-benzanthracene. Am. J. Cancer, 33, 499—537.

17 Sims, P., Grover, P.L., Swaisland, A., Pal, K. and Hewer, A. (1974) Metabolic activations of benzo(a)pyrene proceeds by a diol-epoxide. Nature (London), 252, 326—328.

18 Slaga, T.J., Gleason, G.L., DiGiovanni, J., Sukumaran, K.B. and Harvey, R.G. (1979) Potent tumor-initiating activity of the 3,4-dihydrodiol of 7,12-dimethylbenz(a)-anthracene in mouse skin. Cancer *Res.*, 39, 1934—1936.

19 Tejwani, R., Nesnow, S. and Milo, G.E. (1980) Analysis of intracellular distribution and binding of benzo(a)pyrene in human diploid fibroblasts. Cancer Letters, 10, 57—65.

20 Witiak, D.T., Inbasekaran, M.N., Cazer, F.D., Daniel, F.B. and Hart, R.W. (1979) A novel synthesis of 1,2-dihydro-7,12-dimethylbenz(a)anthracene: a key intermediate leading to the synthesis of proposed A-ring ultimate carcinogenic metabolites of DMBA. Abstract, 177th National Meeting of the American Chemical Society, Honolulu, Hawaii.

21 Yang, L.L., Maher, V.M. and McCormick, J.J. (1980) Excision repair of the N^2-guanine adduct of "anti" 7,8-diol-9,10-epoxide of benzo(a)pyrene by human cells is error free. Fed. Proc., 39, 1738.


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